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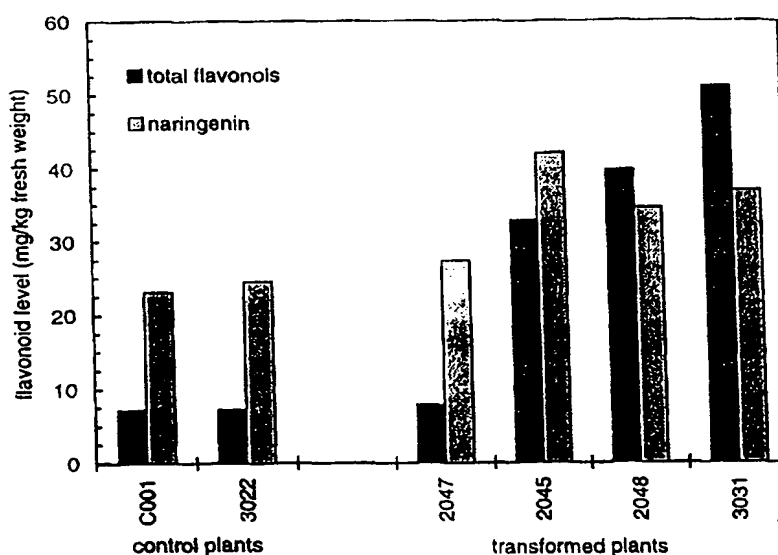
(57) Abstract

    A method for manipulating the production of flavonoids other than anthocyanins in plants by manipulating gene activity in the flavonoid biosynthetic pathway by expressing two or more genes encoding transcription factors for flavonoid biosynthesis, compositions for use in such a method and tomato plants having altered flavonoid levels are disclosed.

METHODS AND COMPOSITION FOR MODULATING
FLAVONOID CONTENT

FIELD OF THE INVENTION

5

The present invention relates generally to methods for manipulating the production of flavonoids in plants by manipulating gene activity in the flavonoid biosynthetic pathway and compositions for use in such methods. In

10 particular, it relates to methods for increasing levels of flavonoids other than anthocyanins by expressing genes encoding transcription factors involved in controlling expression of genes encoding enzymes of the flavonoid biosynthetic pathway.

15 BACKGROUND OF THE INVENTION

Flavonoids form a large group of polyphenolic compounds, based on a common diphenylpropane skeleton, which occur naturally in plants. Included within this class of compounds are flavonols,

20 flavones, flavanones, catechins, anthocyanins, isoflavonoids, dihydroflavonols and stilbenes. The flavonoids are mostly present as glycosides.

In tomato fruits, the main flavonoid found is narichalcone

25 (naringenin chalcone) (Hunt et al, Phytochemistry, 19, (1980), 1415-1419). It is known to accumulate almost exclusively in the peel and is simultaneously formed with colouring of the fruit. In addition to naringenin chalcone, glycosides of quercetin and, to a lesser extent, kaempferol are also found in

30 tomato peel.

Reports in the literature suggest that there is increasing evidence that flavonoids, especially flavonols are potentially health-protecting components in the human diet.

35 Epidemiological studies suggest a direct relationship between cardioprotection and increased consumption of flavonoids, in

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particular flavonols of the quercetin and kaempferol type, from dietary sources such as onion, apples and tea (see, for example, Hertog et al, Lancet, 342 (1993), 1007-1011).

5   Flavonoids have been reported to exhibit a wide range of biological activities *in vitro* including anti-inflammatory, anti-allergic and vasodilatory activity (Cook et al, Nutritional Biochemistry, 7, (1996), 66-76). Such activity has been attributed in part to their ability to act as
10   antioxidants, capable of scavenging free radicals and preventing free radical production. Within this group of compounds, those having the most potent antioxidant activity are the flavonols (Rice-Evans et al, Free Radical Research, 22, (1995), 375-383). In addition, flavonoids can also inhibit the
15   activity of key processes such as lipid peroxidation, platelet aggregation and capillary permeability (see Rice-Evans et al, Trends in Plant Science, 2, (1997), 152-159).

Based on studies of this type, there is presently considerable
20   interest in the development of food products from plants rich in such protective flavonoids.

It would be desirable to produce plants which intrinsically possess elevated levels of health protecting compounds such as
25   flavonoids in order to develop food products with enhanced protective properties. Traditionally, the approach to improving plant varieties has been based on conventional cross-breeding techniques, but these are slow as they require time for breeding and growing successive plant generations. More
30   recently, recombinant DNA technology has been applied to the general problem of modifying plant genomes to produce plants with desired phenotypic traits. Whilst reference has been made in the literature to the use of genetic manipulation techniques in modifying the flavonoid biosynthetic pathway, as discussed
35   beneath, it is notable that these attempts have been directed in general towards modifying pigmentary anthocyanin production.

For example, several studies have attempted to modify the flavonoid pathway by the introduction of genes encoding for enzymes in the flavonoid pathway. Examples of these are

5   EP522880, WO 90/11682, Goldsbrough et al in Plant Physiology (1994) 105:191-194 and Yoder et al, in Euphytica (1994) 79: 163-167.

Other studies have attempted to modify the anthocyanin

10   biosynthesis pathway by altering the expression of a single transcription factor. Examples of these are WO 91/2059, Goldsbrough et al, (1996), Plant Journal, 9(6), 927-933, Mooney et al (1995), Plant Journal (1995), 7(2), 333-339, WO93/14211, WO93/18171 and Moyano et al, in Plant Cell, Vol 8, 1519-1532,

15   1996.

The flavonoid biosynthetic pathway is well established and has been widely studied in a number of different plant species (see, for example, Koes et al, BioEssays, 16, (1994), 123-132).

20   Briefly, three molecules of malonyl-CoA are condensed with one molecule of coumaroyl-CoA, catalysed by the enzyme chalcone synthase, to give naringenin chalcone which rapidly isomerises, catalysed by chalcone isomerase, to naringenin. Subsequent hydroxylation of naringenin catalysed by flavanone 3-

25   hydroxylase leads to dihydrokaempferol. Dihydrokaempferol itself can be hydroxylated to produce either dihydroquercetin or dihydromyricetin. All three dihydroflavonols subsequently can be converted to anthocyanins (by the action of dihydroflavonol reductase and flavonoid glucosyltransferase) or

30   alternatively converted to flavonols such as kaempferol, quercetin and myricetin by the action of flavonol synthase.

Hitherto, studies in maize have identified two regulatory genes, C1 and R which are required for the production of

35   anthocyanin (see Lloyd et al, Science, (1992), 258, 1773-1775). The C1 gene encodes a protein which has a myb DNA binding

domain (Paz-Ares, et al, (1987) EMBO Journal, 6, 3553-3558) whilst the R gene encodes a protein with a basic helix-loop-helix domain characteristic of the myc family of transcriptional regulators (Ludwig et al, Proc. Natl. Acad.

5   Sci., USA, 86 (1989) 7092-7096).

In Lloyd et al, referred to above, the expression of these anthocyanin pathway-specific transcriptional factors from the monocot maize in the dicots *Arabidopsis thaliana* and *Nicotiana*

10   *tabacum* is disclosed.   It is reported that anthocyanin production in both plant species is activated by R (*Lc* allele) in those tissues that normally produce anthocyanins but that *C1* alone has no effect.   Hybrid transgenic *Arabidopsis* expressing both transcription factors, placed under transcriptional

15   control of the cauliflower mosaic virus 35S promoter, was reported to produce anthocyanins in tissues which would not normally express anthocyanins such as root, petal and stamen.

Crosses were performed using one R(*Lc*)-expressing line to

20   pollinate three plants expressing *C1*. As all four parents were heterozygous, it would be expected that one in four of the progeny would contain both R and *C1*. In one cross, thirty six progeny were produced, of which four plants displayed anthocyanin accumulation in the roots and gave a small amount

25   of anthocyanin in the petal and stamen tissue, the exact magnitude of the fold increase over wild type being unrecorded. Of the thirty eight progeny resulting from another cross, three plants gave anthocyanin in the petal tissue whereas in a third cross, no progeny with pigmentation in root or petal tissue

30   were produced. Plants producing anthocyanins in the root and petals are assumed to contain both *C1* and R, although this is not confirmed experimentally, nor is there any explanation as to why none of the progeny resulting from the third cross displayed anthocyanin accumulation in roots and petals. The

35   authors of the study do not report whether the presence of both R and *C1* in *Arabidopsis* leads to the constitutive production of

anthocyanin in the whole plant or if anthocyanin production is restricted to certain areas of the plant.

Reports in the literature suggest that the introduction of a transcription factor from another species into *Arabidopsis* may cause it to behave atypically with respect to upregulation of anthocyanin production, (see for example, Mooney et al, Plant Journal, (1995), 7, 333-339). Here, overexpression of the gene encoding the *Antirrhinum DELILA* transcription factor under the control of the cauliflower mosaic virus 35S promoter gave rise to enhanced anthocyanin levels in both tomato and tobacco but in *Arabidopsis*, no obvious phenotype occurred. In tomato, increased pigmentation was produced in hypocotyl, cotyledon, leaves, stem and roots but no detectable enhancement of normal pigmentation in tomato fruits and testa of the seed was found. Enhanced pigmentation was seen in the flowers in tobacco but no vegetative parts were pigmented.

As reported by Goldsbrough et al, (1996) Plant Journal, 9(6), 927-933, expression of the *Lc* gene in tomato under the control of the cauliflower mosaic virus 35S promoter led to accumulation of anthocyanin only in those tissues which would normally be expected to produce anthocyanins such as leaves, stems, sepals, and the main vein of petals. In leaves, all the anthocyanin production occurred in the epidermal layer only. It is further reported that overexpression of homozygous *Lc* is lethal to the plant.

Quattrocchio et al, in Plant Cell, Vol 5, 1497-1512 (1993) describes the introduction of genes into parts of petunia leaves by particle bombardment. In these experiments the combination of the *Lc* and *C1* transcription factors leads to anthocyanin accumulation in the leaves. No teaching is provided as to what the effects of this methodology are on the production of flavonoids other than anthocyanin such as flavonols. Furthermore it is well known in the art that

experiments carried out by particle bombardment techniques cannot be used as reliable predictors for the effects which could be obtained by transgene incorporation of genes.

5   There are no reports in the literature which confirm that levels of anthocyanins are directly correlated to the levels of flavonols or other flavonoids. Also there is no disclosure in the literature of the manipulation of flavonoids other than anthocyanins in plants by means of expression of
10 transcriptional regulatory factors.

Accordingly, there remains a continuing need for the development of methods for enhancing the levels of flavonoids other than anthocyanins, in particular flavonols, in plants.
15

Furthermore there is a need to enhance the levels of flavonoids other than anthocyanins in plants or specific parts thereof, while avoiding a substantial increase in anthocyanin production, such that on the one hand the amount of desirable
20 ingredients is increased, but the colour of the plant remains substantially the same.

SUMMARY OF THE INVENTION

25 In a first aspect, the invention provides a method for producing a plant capable of exhibiting altered levels of flavonoids other than anthocyanins comprising incorporating into said plant two or more genes each encoding a different transcription factor for flavonoid biosynthesis, or a sequence
30 functionally equivalent thereto, each gene being operably linked to a promoter.

Surprisingly it has been found that a combination of two or more genes encoding for transcription factors provides altered
35 levels of flavonoids other than anthocyanins. Preferably the plants according to the invention are stably transformed with

these genes.

The invention also provides a plant having two or more transgenes each encoding a different transcription factor for
5   flavonoid biosynthesis, or a sequence functionally equivalent thereto, preferably stably incorporated into its genome such that its ability to produce flavonoids other than anthocyanins is altered.

10   The invention further provides a tomato plant having a combination of two or more additional genes encoding transcription factors for flavonoid biosynthesis incorporated, preferably stably, into its genome such that its ability to produce flavonoids other than anthocyanins is altered.
15

Also provided is a transformed plant having enhanced levels of flavonoids other than anthocyanin, particularly flavonol levels, compared to similar untransformed plants.

20   Further provided is a fruit-bearing plant, particularly a tomato plant, having flavonoids, particularly flavonols, in the flesh of the fruit.

Seeds, fruits and progeny of such plants and hybrids are also
25   included within the invention.

The invention further provides a DNA construct comprising sequences coding for a combination of two or more genes, each gene encoding a different transcription factor for flavonoid
30   biosynthesis, or a functionally equivalent sequence thereof, each gene being operably linked to a promoter. When transformed into a plant cell, these constructs are useful in overexpressing genes encoding enzymes of the flavonoid biosynthetic pathway, thereby altering the ability of the plant
35   to produce flavonoids other than anthocyanins. The invention also provides for plants comprising these constructs together

with seeds, fruits and progeny thereof.

Food products such as sauces, dressings, ketchups and soups, comprising a plant prepared according to the invention are also
5 provided.

Also provided are skin and hair protective products comprising a plant according to the invention.

10 BRIEF DESCRIPTION OF THE DRAWINGS

The present invention may be more fully understood by reference to the following description, when read together with the accompanying drawings in which:
15

Figure 1   shows restriction maps of plasmids pUCAP, PUCM2 and pUCM3.

Figure 2   shows restriction maps of plasmids pGPTV-KAN and
20   pBBC3.

Figure 3   shows the T-DNA region of chimeric gene constructs (a) pBBC10; (b) pBBC20; (c) pBBC30; (d) pBBC200; (e) pBBC300
25   Abbreviations: Pnos: nopaline synthase promoter; Pd35s: Cauliflower Mosaic Virus (CaMV) double enhanced 35s promoter; Tnos: nopaline synthase terminator; Trbc: pea ribulose bisphosphate carboxylase small-subunit gene terminator; *C1*: maize
30   *C1* gene; *Lc⁻*: maize *Lc* gene without leader sequence; *Lc⁺*: maize *Lc* gene plus leader sequence.

Figure 4   shows a Southern blot of chromosomal DNA from tomato. Chromosomal DNA was isolated from leaves of
35   transgenic and non-transgenic tomato plants. 5µg DNA was digested with *BglII*, separated on an agarose

gel and blotted to a nylon filter. The DNA was hybridised with a radiolabeled *npt*II specific probe and autoradiographed, using a bio-imager.

5   Figure 5   shows a Southern blot of chromosomal DNA from tomato. Chromosomal DNA was isolated from leaves of transgenic and non-transgenic tomato plants. 5 μg DNA was digested with *Eco*RI (panel A) and *Nco*I (panel B), separated on an agarose gel and blotted
10   to a nylon filter. The DNA was hybridised with a radiolabeled *C1* specific probe and autoradiographed. The arrow points to the hybridising 1.5 kb *Nco*I-band from pBBC10.

15 Figure 6   shows a Southern blot of chromosomal DNA from tomato. Chromosomal DNA was isolated from leaves of transgenic and non-transgenic tomato plants. 5 μg DNA was digested with *Bgl*II/*Cla*I, separated on an agarose gel and blotted to a nylon filter. The DNA
20   was hybridised with a radiolabeled *npt*II specific probe and autoradiographed.

    Figure 7   shows a Southern blot of chromosomal DNA from tomato. Chromosomal DNA was isolated from leaves
25   of transgenic and non-transgenic tomato plants. 5 μg DNA was digested with *Bgl*II/*Cla*I, separated on an agarose gel and blotted to a nylon filter. The DNA was hybridised with a radiolabeled *Lc* specific probe and autoradiographed.
30
    Figure 8   shows typical HPLC chromatograms, recorded at 370nm, of hydrolysed extracts of (A) peel and (B) flesh tissue from red fruits of untransformed tomato plants. Peaks of quercetin and kaempferol aglycons
35   are indicated.

Figure 9   shows typical HPLC chromatograms, recorded at 360nm,
              of non-hydrolysed extracts of (A) peel and (B) flesh
              tissue from red fruits of untransformed tomato
              plants. Peaks of different flavonol-glycosides and
5   of naringenin-chalcone are indicated by compound
              name.

Figure 10   shows HPLC-chromatograms, recorded at 370nm, of
              hydrolysed extracts of flesh tissue from red fruits
10   of (A) an untransformed plant and (B) a plant
              transformed with the pBBC300 gene construct.

Figure 11   shows levels of quercetin, kaempferol and naringenin
              (in mg/kg dry weight (DW)) in extracts from flesh of
15   red fruits from some control plants and plants
              successfully transformed with either the pBBC200
              (2000-2499) series of transformed plants) or the
              pBBC300 (3000 series of transformed plants) gene
              construct. Data were calculated from hydrolysed
20   extracts, i.e. naringenin is derived from
              isomerization of narichalcone and quercetin and
              kaempferol aglycons are derived from their
              respective glycosides.

25 Figure 12   shows levels of total flavonols (quercetin plus
              kaempferol) and naringenin in extracts from whole
              fruits of some control and transformed tomato
              plants. Data were calculated from hydrolysed
              extracts, i.e. naringenin is derived from
30   isomerization of narichalcone, and quercetin and
              kaempferol aglycons are derived from their
              respective glycosides.

Figure 13   shows typical HPLC chromatograms, recorded at 360nm,
35   of non-hydrolysed extracts of flesh tissue from red
              fruits of (A) an untransformed plant and (B) a plant

transformed with pBBC300.

Figure 14   shows   the   ripening-dependent   accumulation   of
             kaempferol-type flavonols in flesh of fruits from a
             plant transformed with pBBC300. HPLC Chromatograms
             recorded at 370 nm of hydrolysed extracts from
             fruits at (A) green, (B) turning and (C) red stage
             are shown.

Figure 15   shows restriction maps of plasmids pFLAP10, pFLAP20
             and pFLAP30.

Figure 16   shows restriction maps of plasmids pFLAP200 and
             pFLAP300.

Figure 17   shows restriction maps of plasmids pBBC10, pBBC20
             and pBBC30.

Figure 18   shows restriction maps of plasmids pBBC200 and
             pBBC300.

Figure 19   shows a restriction map of plasmid pT7E8.

Figure 20   shows a restriction map of plasmid pBBC250.

Figure 21   shows the expression of the regulatory genes *Lc* and
             C1, relative to the constitutive gene *cyp*, in red
             fruits of a control plant (number 004), of plants
             transformed with the single gene constructs pBBC10
             (numbered 100 onwards), pBBC20 (numbered 200
             onwards) or pBBC30 (numbered 300 onwards), and of
             plants transformed with the double gene constructs
             pBBC250 (numbered 2500 onwards) or pBBC300 (number
             3031).   For each plant, the level of kaempferol
             measured in the fruits is given as well. ---: <2
             mg/kg fresh weight; +: 2-10 mg/kg fresh weight: ++:

11-40 mg/kg fresh weight; +++: >40 mg/kg fresh
weight.

Figure 22   shows the relation between total flavonoid level
(quercetin, kaempferol and naringenin) and
antioxidant activity (TEAC values) of red fruits of
some control (c) and pBBC300-transformed (t) tomato
plants.

Figure 23   shows serum pellet ratios for tomato pastes
prepared from transformed tomatoes as compared to
pastes prepared from control tomatoes.

Figure 24   shows a comparison of Bostwick values of tomato
pastes prepared from transformed tomatoes as
compared to pastes prepared from control tomatoes.

Figure 25   shows a spectrum-index plot of a detail of a HPLC
chromatogram, recorded at 280nm, of non-hydrolysed
extracts from whole red fruits of a pBBC300-
transformed plant.   The retention times and
absorbance spectra of flavonoid species that were
increased in the transformed plant are indicated in
the upper panel.

Figure 26   shows typical HPLC chromatograms, recorded at
360nm, of non-hydrolysed extracts from whole red
fruits of (A) an untransformed plant and (B) a
plant transformed with pBBC300. Retention times
and names of identified peaks are indicated.

Figure 27   shows typical HPLC chromatograms, recorded at
280nm, of non-hydrolysed extracts from whole red
fruits of (A) an untransformed plant and (B) a
plant transformed with pBBC300. Retention times
and names of identified peaks are indicated.

Figure 28   shows the levels of total flavonols (quercetin plus
             kaempferol) in hydrolysed extracts of whole red
             fruits of untransformed control plants (left bar,
5   mean ± s.d., n = 10), plants transformed with the
             single gene-constructs pBBC10 (numbered 100
             onwards), pBBC20 (numbered 200 onwards) and pBBC30
             (numbered 300 onwards), as well as some plants
             transformed with the double gene-constructs pBBC200
10   (numbered 2000-2499) and pBBC300 (numbered 3000-
             3499).

Figure 29   shows levels of total flavonols (quercetin plus
             kaempferol), kaempferol, quercetin and naringenin
15   measured by HPLC in whole fruits of control and
             transformed tomato plants. Flavonoid levels are
             expressed in mg/kg fresh weight and were calculated
             from hydrolysed extracts, i.e. naringenin is
             derived from hydrolysis of naringenin-glycosides
20   (in transformants only) and from isomerization of
             narichalcone (both controls and transformants).
             First bar in each plot represents the levels of
             control plants (means ± s.d., n = 10).

25 Figure 30   shows typical GC-MS chromatograms, total ion counts
             (TIC) recorded, of volatiles produced by red tomato
             fruits of (A) an untransformed control plant, (B) a
             plant transformed with pBBC20, (C) a plant
             transformed with pBBC10 and (D) a plant transformed
30   with pBBC200. Arrow indicates the position (with
             integrated peak areas) of methylsalicylate. Y-
             axis: percentage TIC (100% = 8 x 10^6); X-axis:
             retention time (minutes).

35 DETAILED DESCRIPTION OF THE INVENTION

As used herein, "plant" means a whole plant or part thereof, or a plant cell or group of plant cells. Preferably however the invention is particularly directed at transforming whole plants and the use of the whole plant or significant parts thereof,

5   such as fruits, leaves or seeds.

A "flavonoid" or a "flavonol" may suitably be an aglycon or a glycoside.

10   A "gene" is a DNA sequence encoding a protein, including modified or synthetic DNA sequences or naturally occurring sequences encoding a protein, and excluding the 5' sequence which drives the initiation of transcription.

15   A "transcription factor" is a protein which can interact with a promoter and thereby influence the level of expression of a gene operably linked to that promoter. A sequence functionally equivalent thereto is any sequence which encodes a protein which has similar functional properties. It will be

20   appreciated that functional equivalent sequences include but ar enot limited to gene fragments which still have similar functional properties and altered genes (e.g. mutated genes) which still have similar functional properties. Preferably there is a high degree of homology (eye-ball method) between

25   the genes and their functional equivalents e.g. at least 60%, more preferred more than 80%, most preferred more than 95%.

"Operably linked to one or more promoters" means the gene, or DNA sequence, is positioned or connected to the promoter in

30   such a way to ensure its functioning. The promoter is any sequence sufficient to allow the DNA to be transcribed. After the gene and promoter sequences are joined, upon activation of the promoter, the gene will be expressed.

35   A "construct" is a polynucleotide comprising nucleic acid sequences not normally associated in nature.

The present invention is based on the unexpected finding that levels of flavonoids other than anthocyanins in plants, particularly edible plants such as tomato plants, can be
5   manipulated by incorporating into the plant two or more genes encoding transcription factors for flavonoid biosynthesis.

Preferably the plants are stably transformed with the two genes encoding transcription factors e.g. by using genetic
10   modification routes.

The method of the invention may be advantageously used for increasing flavonoid levels other than anthocyanins in a broad variety of plants.   Preferably the invention its applied to
15   edible plants, more in particular food plants which are normally used for food purposes.   Examples of food plants are vegetables such as a tomato plant, spinach, a pea plant, broccoli, cauliflower, asparagus and potato plant, fruit-bearing plants such as a strawberry plant, oil producing plants
20   such as sunflower, soybean and rape, or extractable plants such as tea plants.

Especially advantageously the present invention can be applied to oil producing plants and vegetables.   Most preferably the
25   method of the invention is applied to tomatoes.

Advantageously, by means of the invention, levels of flavonoids, more particularly flavonols, in plants, may be increased.   Typically it is preferred that the increase of
30   flavonoids other than anthocyanins is apparent in at least a part of the plants.   For example for vegetables it is preferred that the flavonoids other than anthocyanins are increased in those parts of the vegetable that are normally eaten.   For oil producing plants the flavonoids other than anthocyanins are
35   preferably increased in the oil producing parts e.g. the sunflower seed or the soy-bean.

Preferably the increase of the levels of flavonoids other than
anthocyanin and/or the increase in the level of flavonols in
the plant as a whole or the desired, normally eaten, part
thereof is increased by at least two-fold, more preferred at
least five-fold, most preferred at least ten-fold as compared
to similar plants which have not been transformed in accordance
to the invention.

Moreover, it has been found that the level of flavonoids other
than anthocyanins, in particular the level of flavonols, may be
increased in specific parts of the plants. For example for
plants of which the leaves are normally eaten or used in food
products such as spinach and tea, it is advantageous that the
level of flavonoids other than anthocyanins, in particular
flavonols are increased in the leaves. For fruit-bearing
plants such as tomato, strawberry etc it is advantageous that
the level is increased in the fruit, for plants with edibe
flowers e.g. broccoli and cauliflower it is advantageous that
the level is increased in the flower, for plants with edible
stems such as asparagus it is advantageous that the level is
increased in the stem, for edible seeds, such as peas,
sunflower seed or rapeseed it is advantageous that the level is
increased in the seed etc. It has been found that typically
the type and choice of one or more of the regulatory sequences
for the genes encoding the transcription factors can provide
the desired increase in specific parts of the plants.

Specifically it has been found that it is possible to increase
the level of flavonoids other than anthocyanins, particularly
flavonols in tomato fruit, and even more surprising in the in
the flesh of tomato fruit, a tissue that does not normally
contain flavonoids, thereby producing tomatoes with enhanced
nutritional, preservative and flavour characteristics.

Preferably the level of flavonols in the flesh of the tomato is

at least 2 mg/kg (fresh weight), more preferred at least 10 mg/kg (fresh weight), most preferably more than 30 mg/kg (fresh weight).

5   A further advantageous embodiment of the invention relates to the increase in the level of flavonoids other than anthocyanins in all or the desired parts of the plant, whereby the level of anthocyanins are not unduly increased such that the colour of the plant or the desired part thereof remains the same.

10

Preferably therefore the level of anthocyanins in the plant as a whole or in the desired part thereof (particularly the fruit) is similar to the level in the untranformed plant, for example the level in transformed plants or parts thereof is less than 2

15   times the level in untransformed plants, more preferably less than 1.5 times, more preferred about the same or less.

Some plants or parts thereof do normally not contain detectable levels of anthocyanins. For example in tomato-plants the peel

20   and flesh of the fruit are normally substantially free from anthocyanins. In preferred embodiments of the invention the level of anthocyanins in such parts, for example the peel and/or the flesh of the tomato fruit, of transformed plants of the invention is therefore equally low e.g. less than 2 mg/kg

25   fresh weight, more preferred less than 1 mg/kg fresh weight, most preferably substantially free from anthocyanins.

It will be appreciated that the invention extends to any plant which is amenable to transformation. Suitable plants include

30   peas, spinach and tea. A particularly preferred plant for use according to the invention is the tomato plant.

Preferably, a combination of genes encoding transcription factors for flavonoid biosynthesis is employed. Suitably, the

35   invention combines the use of a gene encoding a protein within the myb family of transcriptional regulators (see Martin et al,

Trends in Genetics, (1997), 13, 67-73) together with a gene
encoding a protein within the *myc* family of transcriptional
regulators (see Ludwig et al, Cell, (1990), 62, 849-851).
Examples of *myb* type transcription factors are *myb*305, *myb*308,
5   *myb*340, P1 from maize and *C1* from maize. Examples of *myc* type
transcription factors are DEL, *Lc*, R from maize and B from
maize.   A particularly suitable combination of genes for use
according to the invention comprises the gene encoding the
maize *C1 myb*-type transcription factor together with the gene
10   encoding the maize *Lc myc*-type transcription factor but it will
be appreciated that the invention is not limited to this
combination and extends to the use of any combination of genes
encoding transcription factors for flavonoid biosynthesis, such
as *myb*305, *myb*340, and Delila from *antirrhinum* (see Mooney et
15   al, above and Moyano et al, Plant Cell, 8 (1996), 1519-1532).

It will be further appreciated that the sequence encoding a
transcription factor for flavonoid biosynthesis may be a
genomic or cDNA clone, or a sequence which in proper reading
20   frame encodes an amino acid sequence which is functionally
equivalent to the amino acid sequence of the transcription
factor encoded by the genomic or cDNA clone. By "functionally
equivalent" is meant any DNA sequence which is capable of
similar biological activity. A functional derivative can be
25   characterised by an insertion, deletion or a substitution of
one or more bases of the DNA sequence, prepared by known
mutagenic techniques such as site-directed mutagenesis. The
functionality can be evaluated by routine screening assays, for
example, by assaying the flavonoid content of the resulting
30   transgenic plant.

Gene sequences encoding transcription factors for flavonoid
biosynthesis for use according to the present invention may
suitably be obtained from plants, in particular higher plants
35   as these generally possess a flavonoid biosynthetic pathway.
Myb and *myc*-type transcription factor genes involved in the

control of flavonoid biosynthesis have been isolated from various plant species such as maize (see, for example, Lloyd et al, above), *antirrhinum* (see, for example, Goodrich et al, Cell, 68, (1992), 955-964) and *Petunia* (see Quattrocchio et al, Plant Cell, 5, (1993), 1497-1512). In the context of the present invention, the maize plant is a particularly preferred source. Alternatively, equivalent genes could be isolated from plant gene libraries, for example by hybridisation techniques with DNA probes based on known transcription factor genes.

The gene sequences of interest will be operably linked (that is, positioned to ensure the functioning of) to one or more suitable promoters which allow the DNA to be transcribed. Suitable promoters, which may be homologous or heterologous to the gene (that is, not naturally operably linked to a gene encoding a transcription factor for flavonoid biosynthesis), useful for expression in plants are well known in art, as described, for example, in Weising et al, (1988), Ann. Rev. Genetics, 22, 421-477). Promoters for use according to the invention may be inducible, constitutive, or tissue-specific or have various combinations of such characteristics.

Preferably at least one of the genes is linked to a promoter which is either non-constitutive and/or tissue specific. It is believed that the use of at least one constitutive promoter is advantageous in that it may prevent the formation of very high (lethal) doses of one or more ingredients in the flavonoid pathway. The use of tissue-specific promoters may equally be advantageous in that they may lead be used to introduce or enhance formation of flavonoids other than anthocyanin in specific desired parts of the plant, for example those parts that are intended to be eaten (e.g. leaf-specific for spinach and tea, seed-specific for oil producing plants and peas, flower specific for broccoli and cauliflower, stem specific for asparagus, fruit specific for tomato).

Useful promoters include, but are not limited to constitutive promoters such as carnation etched ring virus (CERV), cauliflower mosaic virus (CaMV) 35S promoter, or more particularly the double enhanced cauliflower mosaic virus

5   promoter, comprising two CaMV 35S promoters in tandem (referred to as a "Double 35S" promoter).

It may be desirable to use a tissue-specific or developmentally regulated promoter instead of a constitutive promoter in

10   certain circumstances. A tissue-specific promoter induces or increases expression of the transcription factor for flavonoid biosynthesis in certain desired tissues, preferably without unduly affecting expression in other tissues. By way of illustration, promoters used in overexpression of the maize *Lc*

15   transcription factor in tomato plants will preferably be tissue specific, especially fruit-specific. Overexpression of *Lc* in vegetative tissues of tomato plants is known to be deleterious to the health of the plant (see Goldsbrough et al, above). Suitable fruit-specific promoters include the tomato E8

20   promoter (Deikman et al, (1988), EMBO J, 7, 3315-3320), 2A11 (Van Haaren et al, Plant Mol Biol, 21, 625-640), E4 (Cordes et al, (1989), Plant Cell, 1, 1025-1034) and PG (Bird et al, (1988), Plant Mol. Biol., 11, 651-662,) Nicholass et.al. (1995), Plant Molecular Biology, 28, 423-435. Both

25   transcription factors for use according to the method of the invention may conveniently operably be linked to the same or different fruit-specific promoters.

According to a preferred embodiment of the method of the

30   invention, however, a gene encoding a first transcription factor for flavonoid biosynthesis is operably linked to a constitutive promoter whilst a gene encoding a second transcription factor for flavonoid biosynthesis is operably linked to a non-constitutive or tissue-specific promoter e.g. a

35   fruit specific promoter. The combination of a constitutive and fruit-specific promoter helps to ensure that the desired

flavonoids are produced mainly in the fruit. An additional advantage in the use of such a combination, is that possible inhibitory effects ("gene silencing") on the expression of the introduced genes arising from the use of the same promoter are
5   avoided.

In a particularly preferred embodiment, a gene encoding the maize *C1* transcription factor operably linked to the constitutive double 35S CaMV promoter is combined with a maize
10   *Lc* transcription factor operably linked to the fruit-specific tomato E8 promoter.

It will be appreciated that overexpression of genes encoding transcription factors for flavonoid biosynthesis according to
15   the method of the invention may result in the accumulation of both anthocyanins and flavonoids other than anthocyanins, particularly if the transcription factors operate at a position along the flavonoid biosynthesis pathway before the respective flavonol and anthocyanin pathways diverge. This may sometimes
20   undesirable as the formation of anthocyanins not only results in the production of aesthetically unpleasing purple coloured fruits, but may also limit the production of flavonols. In a preferred embodiment of the invention, therefore those genes for encoding transcription factors are chosen which on the one
25   hand lead to an increase of the level of flavonoids other than anthocyanin in the whole plant or the desired tissues, but on the other hand do not substantially enhance the level of anthocyanins in said plant or the desired tissues thereof. In a further embodiment, this may be accomplished by blocking the
30   route to expression of anthocyanins for example by an additional step comprising antisense suppression of dihydroflavonol reductase (DFR), the enzyme catalysing the final step in the production of anthocyanins. Alternatively, transcription factors may be overexpressed in a mutant line,
35   such as a tomato line which is deficient in DFR activity, for example, the anthocyanin without (aw) mutant which is described

by Goldsbrough et al, Plant Physiol, (1994), 105, 491-496.

Furthermore, accumulation of flavonoids may also be inhibited
by the rate of production of the amino acid phenylalanine, the
5   primary substrate in the synthesis of phenylpropanoids and
subsequent flavonoids.   In order to increase phenylalanine
biosynthesis, genes encoding enzymes of the phenylalanine
pathway that are insensitive to feed-back regulation may be
introduced as an optional additional step.
10

Plants incorporating a combination of genes encoding
transcription factors for flavonoid biosynthesis according to
the invention may be produced by crossing one plant expressing
one transcription factor of the pair with another plant
15   expressing the other transcription factor using conventional
cross-breeding techniques.   The respective starting materials
may be produced by conventional plant transformation techniques
well known in the art.

20   Preferably, however, the desired gene sequences, operably
linked to respective suitable promoters, are fused to
appropriate expression sequences to provide an expression
cassette functional in a plant cell which can be introduced
into a plant cell by any conventional plant transformation
25   method.

Accordingly, the invention provides in a further aspect an
expression cassette comprising as operably linked components in
the 5'-3' direction of transcription, two or more units each
30   comprising a promoter functional in a plant cell, a gene
encoding a transcription factor for flavonoid biosynthesis and
a transcriptional and translational termination regulatory
region functional in a plant cell.

35   The promoter and termination regulatory regions will be
functional in the host plant cell and may be heterologous (that

is, not naturally occurring) or homologous (derived from the plant host species) to the plant cell and the gene. Suitable promoters which may be used are described above.

5   The termination regulatory region may be derived from the 3' region of the gene from which the promoter was obtained or from another gene. Suitable termination regions which may be used are well known in the art and include *Agrobacterium tumefaciens nopaline synthase terminator (Tnos), Agrobacterium tumefaciens*
10   *mannopine synthase terminator (Tmas)* and the *CaMV 35S terminator (T35S)*. Particularly preferred termination regions for use according to the invention include the tobacco ribulose bisphosphate carboxylase small subunit termination region (TrbcS) or the *Tnos* termination region.
15

       Such gene constructs may suitably be screened for activity by transformation into a host plant via *Agrobacterium* and screening for flavonoid levels.

20   Conveniently, the expression cassette according to the invention may be prepared by cloning the individual promoter/gene/terminator units into a suitable cloning vector. Suitable cloning vectors are well known in the art, including such vectors as pUC (Norrander et al, (1983, Gene 26, 101-106),
25   pEMBL (Dente et al (1983), Nucleic Acids Research, 11, 1645-1699), pBLUESCRIPT (available from Stratagene), pGEM (available from Promega) and pBR322 (Bolivar et al, (1977), Gene, 2, 95-113). Particularly useful cloning vectors are those based on the pUC series. The cloning vector allows the DNA to be
30   amplified or manipulated, for example by joining sequences. The cloning sites are preferably in the form of a polylinker, that is a sequence containing multiple adjacent restriction sites, so as to allow flexibility in cloning.

35   In a particularly preferred embodiment, the individual promoter/gene/terminator units are cloned into adjacent pairs

of restriction sites in a suitable cloning vector. The
individual promoter/gene/terminator units may conveniently be
constructed using a cloning vector comprising the same
restriction sites as are present in the cloning vector for the
5   multiple unit construct but wherein the restriction sites are
arranged in nested fashion rather than sequentially.

Suitably, the nucleotide sequences for the genes may be
extracted from the Genbank nucleotide database and searched for
10   restriction enzymes that do not cut. These restriction sites
may be added to the genes by conventional methods such as
incorporating these sites in PCR primers or by sub-cloning.

Preferably the DNA construct according to the invention is
15   comprised within a vector, most suitably an expression vector
adapted for expression in an appropriate host (plant) cell. It
will be appreciated that any vector which is capable of
producing a plant comprising the introduced DNA sequence will
be sufficient.
20

Suitable vectors are well known to those skilled in the art and
are described in general technical references such as Pouwels
et al, Cloning Vectors. A laboratory manual, Elsevier,
Amsterdam (1986). Particularly suitable vectors include the Ti
25   plasmid vectors.

Transformation techniques for introducing the DNA constructs
according to the invention into host cells are well known in
the art and include such methods as micro-injection, using
30   polyethylene glycol, electroporation, or high velocity
ballistic penetration. A preferred method for use according to
the present invention relies on agrobacterium - mediated
transformation.

35   After transformation of the plant cells or plant, those plant
cells or plants into which the desired DNA has been

incorporated may be selected by such methods as antibiotic
resistance, herbicide resistance, tolerance to amino-acid
analogues or using phenotypic markers.

5   Various assays may be used to determine whether the plant cell
     shows an increase in gene expression, for example, Northern
     blotting or quantitative reverse transcriptase PCR (RT-PCR).
     Whole transgenic plants may be regenerated from the transformed
     cell by conventional methods. Such transgenic plants having
10   improved flavonoid levels may be propagated and crossed to
     produce homozygous lines. Such plants produce seeds containing
     the genes for the introduced trait and can be grown to produce
     plants that will produce the selected phenotype.

15   In accordance with a particular embodiment of the invention,
     the cloning vectors plasmid pUCM2 and pUCM3 were prepared by
     modifying the cloning vector pUCAP (Van Engelen et al, (1995),
     Transgenic Research, 4, 288-290). Multiple cloning sites in
     these plasmids were constructed by insertion of synthetic
20   adapters containing the restriction sites needed. Each
     individual gene construct encoding a transcription factor for
     flavonoid biosynthesis is prepared in pUCM2. Generally, each
     promoter was cloned as a KpnI/BamHI fragment, each structural
     gene as a BamH1/Sall fragment and each terminator region as a
25   Sall/Clal fragment, such that each individual gene fusion was
     made as a Kpn1/Clal fragment. By using restriction sites
     outside of Kpn1 and Clal the individual gene fusions can be
     cloned behind each other in plasmid pUCM3, which contains the
     restriction sites of pUCM2 but in a different order. Using the
30   two unique restriction sites PaC1 and AsC1, the desired
     multigene constructs were transformed to the plant
     transformation binary vector plasmid pBBC3, a derivative of
     plasmid pGPTV-KAN to which a synthetic adaptor containing a
     unique PaC1 and AsC1 restriction site had been added.

35

     The invention also relates to the use of the plants according

to the invention or desired parts thereof in the preparation of food products or skin or hair protective products.

For example for the preparation of food products the desired
5   parts of the plant with the altered level of flavonoids other than anthocyanin may be harvested and further processes into an edible product.

For example leaves of spinach may be harvested and heated (e.g.
10   by blanching) optionally comminuted and subsequently frozen to produce a frozen spinach product with altered levels of flavonoids other than anthocyanins. Similarly tea leaves can be processed into leaf tea or filled into tea-bags to provide tea with enhanced levels of flavonoids other than anthocyanins.
15

In another embodiment seeds, e.g. pea seed may be harvested and further processed e.g. blanching and freezing into pea products. Also comminuted products may be made e.g. pea-soup. Other seeds e.g oil seeds such as sunflower seed or soy-bean
20   may be used for the extraction of oil, preferably the conditions of oil extraction and further processing (e.g refining, esterification etc) are chosen such that the final oil still possesses the enhanced levels of flavonoids other than anthocyanins.
25

In another embodiment flowers such as broccoli or cauliflower may be harvested and further processed e.g. to prepare frozen vegetables or soup. Also stems such as asparagus may be harvested and further processed e.g. to product asparagus soup.
30

A particulare preferred embodement of the invention relates to the use of fruits especially of tomatoes with increased levels of flavonoids other than anthocyanins, particularly flavanols. These tomatoes may be harvested and eaten as such.
35   Alternatively the tomatoes may be used in the preparation of food products. For example parts of tomato may be added to

salads. Also heat-treatment may be applied, for example tomatoes may be used to prepare tomato sauces with tomato as one of the main ingredients (e.g. at levels of 10% by weight or more, for example 80% by weight or more) such as tomato paste,

5   tomato ketchup, pizza sauce, pasta sauce, dressings etc. Also the tomatoes may be used to prepare products like tomato juice, tomato soups etc.

Surprisingly it has been found that plants of the invention not

10   only have altered levels of flavonoids other than anthocyanins but also have other advantages.

One particular advantage is that plant of the invention, in particular tomatoes may have enhanced levels of

15   methylsalicylate. This is a well-known precursor in the flavour pathway and hence leads to a better flavour of the products of the invention.

A further embodiment of the invention is that plants of the

20   invention, especially tomatoes may lead to improved rheological properties of the products produced therefrom. For example when tomato sauces such as tomato paste, tomato ketchup, pizza sauce, pasta sauce, dressings etc are made from tomatoes of the invention this may lead to an increase of the thickness of said

25   sauces.

The following examples are provided by way of illustration only.

30   DNA manipulations were performed using standard procedures well known in the art, as described, for example, in Sambrook et al, Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbour Laboratory Press, 1989 (hereinafter "Sambrook").

35   The following literature references are mentioned in the Examples:

Becker, D. et al. (1992) Plant Mol. Biol. 20: 1195-1197.

Bonierbale, M.W. et al. (1988) Genetics 120: 1095-1103.

Bovy, A.G. et al. (1995) Acta Hortic. 405: 179-189.

Damiani, R. et al. (1993) Proc. Natl. Acad. Sci. USA 90: 8244-8248.

Hanahan, D. (1983) J. Mol. Biol. 166: 557-580.

Hertog, M.G.L. et al. (1992) J. Agric. Food Chem. 40: 1591-1598.

Hoekema, A. et al. (1985) Plant Mol. Biol. 5: 85-89.

Jackson , D. et al. (1991) Plant Cell 3: 115-125.

Jefferson, R. et al. (1987) Embo J. 6: 3901-3907.

Lloyd,A. et al. (1992) Science 258:1773-1775.

Miller, N.J. and Rice-Evans C.A. (1997) Free Rad. Res. 26: 195-199.

Murashige, T. and Skoog, F. (1962) Physiol. Plant. 15: 73-97.

Van Engelen, F. et al. (1995) Transgenic R. 4: 288-290.

Verhoeven, H.A. et al (1997) Chromatographia 46: 63-66.

EXAMPLES

Example 1: Plant material

All experiments can be performed using normally available processing tomato lines as the starting material. FM6203 is such a line which is comparable to the commercially available lines such as var.Napoli and var.Roma VF which are available from Simpson's Seeds (Tomato Growers Club, Surrey, England, MAFF registration number 2620).

Tomato line FM6203 was grown in a greenhouse with a 16h photoperiod and a 21/17°C day/night temperature.

Example 2: Bacterial strains

The Escherichia coli strain used was:

DH5' *supE*44,   D(*lacZYA-ArgF*)U169,   f80*lac*ZDM15,   *hsd*R17   (r_k-,
m_k+), *rec*A1, *end*A1, *gyr*A96, *thi*-1, *rel*A1, *deo*R (Hanahan, 1983).

5   The Agrobacterium strain used was LBA4404 (Hoekema, 1985).

Transformation of E. coli DH5' was performed using the method
of Hanahan (1983).

10   Transformation of Agrobacterium LBA4404 was performed according
to Gynheung et al (1988) in Plant Molecular Biology Manual,
Eds. Gelvin and Schilperoort, Kluwer Academic Publishers
(Dordrecht) pPMAN-A311-19.

15   **Example 3: Gene constructs**

   **3.1   Strategy to overexpress flavonoid biosynthesis genes in
          tomato fruits**
   The production of flavonoids in tomato fruits is increased by
20   over-expression of the maize transcription factor-genes *C1* and
   Lc, whose gene-products are capable of inducing the expression
   of the endogenous tomato flavonoid biosynthesis genes.   To
   increase the level of flavonoids predominantly in the fruit of
   tomato the *Lc* gene is expressed under control of the fruit
25   specific tomato E8 promoter.   The *C1* gene is expressed under
   control of the constitutive CaMV 35s promoter with double
   enhancer.

   A cassette approach was used to clone the different components
30   of the gene constructs, so that all promoters were cloned with
   the same restriction enzymes, all structural genes with the
   same restriction enzymes and all terminators with the same
   restriction enzymes.   To select such restriction enzymes the
   nucleotide sequences for all the above mentioned genes were
35   extracted from the Genbank nucleotide database and were
   searched for restriction enzymes that do not cut.   These

restriction sites were added to the genes either by incorporating these sites in PCR primers, or by subcloning. The genes were then cloned step by step in specially designed pUC-derived vectors that contain the selected restriction
5 enzyme sites in their multiple cloning site.

The inserts of the final constructs were transferred to a plant transformation vector and transformed into tomato.

10 **3.2   Construction of cloning vectors pUCM2 and pUCM3**
Plasmids pUCM2 and pUCM3 were derived from the cloning vector pUCAP (Figure 1; Van Engelen et al. (1995)). The multiple cloning sites in these plasmids were constructed by insertion of synthetic adapters containing the restriction sites needed.
15 First, each gene construct is made in pUCM2. All promoters are cloned as a *Kpn*I/*Bam*HI fragment, each structural gene as a *Bam*HI/*Sal*I fragment and each terminator as a Sal I/Cla I fragment. So all gene fusions are made as a *Kpn*I/*Cla*I fragment. By using the restriction sites outside of *Kpn*I and
20 *Cla*I, the gene fusions can be put behind each other in plasmid pUCM3, which contains the restriction sites of pUCM2 in a different order. With these two gene cassettes up to four gene fusions can be cloned behind each other in one construct. With the two unique restriction sites *Pac*I and *Asc*I the multigene
25 constructs can be transferred to binary vector pBBC3 (Figure 2), a derivative of pGPTV-KAN (Becker et al. (1992)).

3.3   Construction of *Lc* and *C1* gene fusions
Following the strategy described above, five binary constructs
30 were made containing fusions of either the *Lc* or *C1* gene alone, or both the *Lc* and *C1* gene together. Two versions of the *Lc* gene were used: an *Lc* cDNA with its 5' untranslated leader (*Lc*$^+$) and an *Lc* cDNA lacking its 5' untranslated leader (*Lc*$^-$). The 5' leader contains a small open reading frame that
35 represses *Lc* translation and hence the highest levels of *Lc*

protein are obtained with the latter *Lc* gene (Damiani, 1993). An overview of the T-DNA region of these constructs is shown in Figure 3. Constructs pBBC10, pBBC20 and pBBC30 are single-gene constructs. Constructs pBBC200 and pBBC300 are two-gene

5   constructs containing both the *C1* and *Lc* genes used to increase the amount of flavonols in tomato fruit (particularly in the flesh).

In construct pBBC*10* the maize *C1* gene was fused with the double

10   enhanced *35s* promoter and the *nos* terminator. Constructs pBBC20 and pBBC30 are single-gene constructs in which both versions of the maize *Lc* gene (minus and plus 5' mRNA leader respectively) are fused with the tomato *e8* promoter and the pea *rbcS* terminator. Construct pBBC200 is a two-gene construct

15   consisting of the *C1* gene fused to the *d35s* promoter and *nos* terminator, and the *Lc* gene (minus leader) fused to the *e8* promoter and *rbcS* terminator. Construct pBBC300 is identical to pBBC200, except that this construct contains the *Lc* gene plus 5' leader. A detailed description of the construction of

20   all plasmids used is given below.

Gene constructs

The different components of the gene fusions and the different plasmid vectors used were obtained as follows. Plasmid vector

25   pUCAP (Van Engelen et al. (1995)) was provided by CPRO-DLO. Binary vector pGPTV-KAN (Becker et al. 1992)) was provided by Unilever. The *nos* terminator (*Tnos*) was amplified from plasmid pBI121 (Jefferson et al. (1987)). The double enhanced CaMV *35s* promoter (*Pd35s*) was isolated from plasmid pMOG18 (Symons et

30   al, (1990), Biotechnology, 8, 217-221). The tomato *e8* promoter (*Pe8*) was PCR amplified from tomato genomic DNA (variety Moneymaker) using Taq polymerase and primers E851 (5' GAATTCAAGCTTGACATCCCTAAT 3') and E8A2 (5' CTTTTGCACTGTGAATGATTAGA 3'). E851 and E8A2 hybridise to the

35   distal (5') and proximal (3') ends of the e8 promoter

respectively. The resulting 2.2 kb PCR fragment was then cloned in the Eco RV site of pT7 Blue-T vector (available from Novagen). This resulted in a vector with the e8 promoter inserted in clockwise orientation (same as lac Z gene), which

5   was called pT7E8 (Figure 16). Constructs containing the *C1* gene (pAL77), the *Lc* gene plus 5' leader (pAL69) and the *Lc* gene minus 5' leader (pAL144) were obtained from R. W. Davis (Stanford University, see also Lloyd et al (1992). The *Lc* genes in plasmids pAL69 and pAL144 were fused to the *rbcS*

10   terminator (*TrbcS*) and could be used as such. All components were cloned step by step as described below.

A.   Construction of plasmids pUCM2 and pUCM3

To construct plasmid pUCM2 the multiple-cloning-site of plasmid

15   pUCAP was modified by the insertion of two adapters. First, adapter F1F2, containing the restriction sites *SalI/ClaI/SphI* (Table 1), was cloned in the pUCAP plasmid digested with *SalI/SphI*. This resulted in plasmid pUCM1. Next, adapter F3F4, consisting of *PacI/NotI/BglII/EcoRI* restriction sites,

20   was cloned in plasmid pUCM1 digested with *PacI/EcoRI*. This resulted in plasmid pUCM2 (Figure 1).

To construct plasmid pUCM3, plasmid pUCAP was digested with *PacI/AscI* and the whole multiple-cloning-site was replaced by

25   adapter F5F6 (Table 1). This resulted in plasmid pUCM3 (Figure 1).

B.   Construction of pBBC3

To construct plasmid pBBC3, adapter F38F39 was ligated in

30   plasmid pGPTV-KAN digested with *EcoRI/HinDIII*. In this way the *gusA-Tnos* gene in pGPTV-KAN is replaced by a small multiple-cloning-site consisting of *PacI/EcoRI/HinDIII/AscI* restriction sites (Figure 2).

35   **C.   Construction of pBBC10**

The *C1* gene fusion was cloned in pUC derivative pUCM2 in three major steps.

Firstly, T*nos* was amplified by PCR from pBI121 with primers F12 and AB13 (see Table 1). The resulting 250bp product was cloned in pUCM2 as a *Sal*I/*Cla*I fragment. This resulted in plasmid pFLAP1.

Table 1:   **Overview of PCR primers and adapters used**

primer(*)	sequence (5' to 3')
F1	TCGACCATATCGATGCATG
F2	CATCGATATGG
F3	TAAGCGGCCGCAGATCTGG
F4	AATTCCAGATCTGCGGCCGCTTAAT
F5	TAAGGGGTACCACCATCGATACCGAATTCTACATGCATGCATGGAGATCT
	CCCAAGCTTCTAAGATGCGGCCGCTAAACATGG
F6	CGCGCCATGTTTAGCGGCCGCATCTTAGAAGCTTGGGAGATCTCCATGCA
	TGCATGTAGAATTCGGTATCGATGGTGGTACCCCTTAAT
F7	AATTGCACCGGTCG
F8	GATCCGACCG
F9	TAGCCATGGG
F10	TCGACCCATGGCTAAT
F12	CCCGTCGACTTTCCCCGATCGTTCAAACATTTGGC
AB13	CCCATCGATGCGTCTAGTAACATAGATGAC
F38	AATTGGGCGCGCCAAGCTTCCGAATTCTTAATTAAG
F39	AGCTCTTAATTAAGAATTCGGAAGCTTGGCGCGCCC
F69	ATGAGAGTGTGAGGAAGGAG
F72	GCCATAATACTCGAACTCAG
F80	TGGGCACAACAGACAATCGGCTGC
F81	TGCGAATCGGGAGCGGCGATACCG
CYP1S	CTTCGCCGATACCACTCCCAAAAC
CYP2A	ACCGCAGTCAGCAATAACCACAGG

* Adapters are made by combining two primers, heating to 95°C

for 5' and anneal both primers by cooling slowly to room temperature.

Secondly, the *C1* gene was cloned as a *Bam*HI/*Sal*I fragment
5   upstream of T*nos* in pFlap2 as follows. The *C1* gene was transferred as a 2kb *Eco*RI fragment from plasmid pAL77 to high-copy plasmid pBluescript SK-, resulting in plasmid pB*LC1*. The *C1* gene was isolated from pBLC1 as a 1.6kb *Eco*RI/*Pac*I fragment and adapters F7F8 and F9F10 (Table 1) were ligated to each end
10   of the fragment in order to add unique *Bam*HI and *Sal*I restriction sites on both ends of the gene and to destroy the *Eco*RI and *Pac*I sites. The resulting *Bam*HI/*Sal*I *C1* fragment was cloned upstream of the nos terminator, resulting in plasmid pFLAP2.
15

Thirdly, P*d35s* was cloned as a *Kpn*I/*Bam*HI fragment upstream of *C1* in pFLAP2 as follows. To create a unique *Bam*HI site at the 3' end of the *d35s* promoter plasmid pMOG18 was digested with *Eco*RV/*Bam*HI, thus removing the 3' part of the *d35s* promoter and
20   the *gusA* gene. The 3' part of the *35s* promoter present in plasmid pAB80 (Bovy et al. (1995)) was ligated as a 0.2kb *Eco*RV/*Bam*HI fragment in the pMOG18 vector, resulting in plasmid pMOG18B. To create a unique *Kpn*I site at the 5' end of the *d35s* promoter plasmid pMOG18B was digested with *Eco*RI, the ends
25   were polished with Klenow polymerase, and subsequently digested with *Bam*HI. The resulting 0.85kb blunt/*Bam*HI *d35s* promoter fragment was cloned into plasmid pB*LC1* digested with *Xho*I/polished with Klenow polymerase/*Bam*HI. This resulted in plasmid pB*l*d35S. Finally the *d35s* promoter was transferred as
30   a *Kpn*I/*Bam*HI fragment from pB*l*d35s to plasmid pFLAP2. This resulted in plasmid pFLAP10 (Figure 15).

The insert of plasmid pFLAP10 was transferred as a 2.8kb *Pac*I/*Asc*I fragment to binary vector pBBC3, resulting in plasmid
35   pBBC10 (Figures 3 and 17).

D.   Construction of pBBC20

The construction of plasmid pBBC20 consists of three major steps: (i) cloning the *Lc⁻* gene (minus leader) plus the *rbcS*
5   terminator in the pUCM2 vector, (ii) cloning the fruit-specific tomato *e8* promoter upstream of the *Lc⁻* gene, and (iii) transfer of the *Lc* fusion to binary vector pBBC3. These steps will be outlined in the following paragraphs.

10   Firstly, the *Lc⁻* gene and the *rbcS* terminator were isolated as a 2.8kb *BamHI/ClaI* fragment from plasmid pAL144 and cloned into plasmid pUCM2 digested with the same enzymes. This resulted in plasmid pFLAP4.

15   Secondly, the tomato *e8* promoter was cloned upstream of the *Lc⁻* gene as follows. The *e8* promoter was present as a 2.2kb fragment on plasmid pT7E8. This *e8* promoter fragment contained an unwanted *PacI* site at position 430 relative to the 5' end. To remove this *PacI* site, plasmid pT7E8 was digested with *PacI*,
20   the ends were polished with T4 DNA polymerase, and the plasmid was self-ligated, resulting in plasmid pT7E8-Pac. The *e8* promoter was subsequently amplified from this plasmid by PCR with primers F23 and F26, which contained unique *KpnI* and *BamHI* restriction sites respectively (Table 1). The PCR product was
25   digested with these enzymes and cloned upstream of the *Lc⁻* gene in plasmid pFLAP4. This resulted in plasmid pFLAP20 (Figure 15).

Thirdly the insert of pFLAP20 was transferred as a 5.1kb
30   *PacI/AscI* fragment to binary vector pBBC3, resulting in plasmid pBBC20 (Figures 3 and 17).

E.   Construction of pBBC30

Plasmid pBBC30 is identical to pBBC20, except for the presence
35   of the *Lc⁺* gene instead of the *Lc⁻* gene. This plasmid was

constructed as follows.

Firstly, the *Lc⁺/TrbcS* gene fusion was cloned as a EcoRI/ClaI
fragment in the pEMBL-derivative pAB10. This resulted in
5   plasmid pAB*LC⁺*.

Secondly, the *Lc⁺/TrbcS* gene fusion was transferred as a
BamHI/ClaI fragment from pAB*LC⁺* to plasmid pFLAP20, thus
replacing *Lc⁻/TrbcS* with *Lc⁺/TrbcS*. This resulted in plasmid
10   pFLAP30 (Figure 15).

Thirdly the insert of pFLAP30 was transferred as a 5.3kb
PacI/AscI fragment to binary vector pBBC3, resulting in plasmid
pBBC30 (Figures 3 and 17).
15

F.   Construction of pBBC200
The single-gene constructs described above were used to
construct plasmids pBBC200 and pBBC300 as follows.

20   Firstly, the *Pd35s-C1-Tnos* insert of plasmid pFLAP10 was
transferred as a 2.8kb *KpnI/ClaI* fragment to plasmid pUCM3,
resulting in plasmid pFLAP100.

Secondly, the *Pe8-Lc⁻-TrbcS* insert of plasmid pFLAP20 was
25   transferred as a 5.1kb *NotI/AscI* fragment to plasmid pFLAP100,
resulting in plasmid pFLAP200 (Figure 16).

Thirdly, the insert of plasmid pFLAP200 is transferred as a
7.9kb *PacI/AscI* fragment to binary vector pBBC3, resulting in
30   plasmid pBBC200 (Figures 3 and 18).

G.   Construction of pBBC300
To construct pBBC300, the *Pe8-Lc⁺-TrbcS* insert of plasmid
pFLAP30 was transferred as a 5.3kb *NotI/AscI* fragment to
35   plasmid pFLAP100, resulting in plasmid pFLAP300 (Figure 16).

The insert of plasmid pFLAP300 is transferred as a 8.1kb *PacI/AscI* fragment to binary vector pBBC3, resulting in plasmid pBBC300 (Figures 3 and 18).

Example 4: Stable transformation of regulatory gene constructs in tomato line FM6203

4.1 A. Tumefaciens transformations

Binary plasmids pBBC10, pBBC20, pBBC30, pBBC200 and pBBC300 were introduced into *Agrobacterium* strain LBA4404 by adding 1μg of plasmid DNA to 100μl of competent Agrobacterium cells, prepared by inoculating a 50ml culture in YEP medium (Sambrook, 1989) and growing at 28°C until the culture reaches an OD_{600} of 0.5-1.0. The cells were then pelleted, resuspended in 1ml of $CaCl_2$ solution and dispensed into 100μl aliquots. The DNA-*Agrobacterium* mixture was frozen in liquid nitrogen and thawed in a water bath at 37°C. After the addition of 1ml YEP medium the bacteria were incubated at 28°C for 4 hours with gentle shaking. Finally transformed bacteria were selected on YEP-agar plates containing 50μg/ml kanamycin. The presence of the plasmids was tested by restriction enzyme analysis.

4.2 Tomato transformations

Seeds from tomato line FM6203 were sterilised by a 2h incubation in 1.5% hypochlorite, followed by three rinses of sterile water. The seeds were germinated and seedlings were grown for 8 days on a 1:1 mixture of vermacolite and MS medium (Murashige and Skoog, 1962; Duchefa) supplemented with 0.3% (w/v) sucrose, with a photoperiod of 16h (3000 lux) at 25°C.

Eight-day old cotyledons were cut into 25mm² squares and preincubated for 24h on tobacco suspension feeder layer plates at low light intensity (1000 lux). The tobacco leaf suspension culture was grown on plates containing MS medium including vitamins, supplemented with sucrose (3% w/v), agarose (6g/l),

2,4-dichlorophenoxyacetic   acid   (2,4-D;   0.5mg/l)   and
benzylaminopurine (BAP; 0.5mg/l).

5   A single colony from the *Agrobacterium* LBA4404 cultures
containing one of the binary vectors mentioned in Examples 3
and 4.1 was grown for 48h in liquid Minimal A medium (Sambrook,
1989) supplemented with 50µg/ml kanamycin to an OD_{600} of 0.5-
1.0.   The bacteria were pelleted by centrifugation and
resuspended in MS medium including vitamins (Duchefa) and 3%
10   (w/v) sucrose at an OD_{600} of 0.5.   The cotyledon explants were
incubated in the *Agrobacterium* suspension for 30', blotted dry
on filter paper and co-cultivated for 48h on tobacco feeder
layer plates at 25°C and low light intensity.

15   After   co-cultivation,   the   explants   were   transferred   to
regeneration medium, consisting of MS medium supplemented with
Nitsch vitamins, sucrose (2% w/v), agargel (5 g/l), zeatin-
riboside (2mg/l), kanamycin (100mg/l) and cefotaxime (500mg/l).
Regenerating explants were transferred to fresh medium every
20   two weeks.   Regenerating kanamycin resistant shoots were
transferred to rooting medium, consisting of MS medium plus B5
vitamins, supplemented with sucrose (0.5% w/v), gelrite (2g/l),
kanamycin   (50mg/l)   and   cefotaxime   (250mg/l).   During
regeneration and rooting explants were incubated in a growth
25   chamber at 25°C with a 16h photoperiod (3000 lux).   After root
formation, plantlets were transferred to soil and grown in the
glasshouse.

Transgenic plants carrying the constructs pBBC200 and pBBC300
30   were numbered from 2001-2499 and 3001-3499 respectively.

Example 5: **Southern analysis of transgenic plants**

5.0   Southern   analysis   of   plants   transformed   with   plasmids
35   **pBBC200 and pBBC300**
The   presence   and   the   copy   number   of   the   transgenes   was

determined in transgenic plants by southern hybridisation.
Genomic DNA was isolated from young leaves as described by
Bonierbale et al.(1988).Aliquots of 5μg genomic DNA were
digested for 16h with *Bgl*II and separated on a 0.7% TAE
5   agarose gel. The DNA was then denatured in 0.4 M NaOH for 30'
and transferred to a Hybond N+ membrane in 0.4M NaOH.

The blots were probed with a 700bp ^{32}P radiolabeled *nptII*-
specific PCR fragment, amplified from plasmid pBBC3 with
10   primers F80 and F81 (Table 1), under stringent conditions
(65°C). Prehybridisation was carried out for 2h at 65°C in a
mix of 6.6 x SSC, 10 x Denhardt's solution, 0.1% SDS, and
0.1mg/ml denatured herring sperm DNA. Hybridisation was
performed by adding denatured probe DNA to the
15   prehybridisation medium and continuing the incubation at 65°C
for 16h. The hybridised blots were washed three times for 30
minutes at 65°C in 0.2 x SSC, 0.1% SDS and autoradiographed
using a bio-imager (Fuji).

20   *Bgl*II cuts in the T-DNA immediately upstream of the *nptII*
gene, 1.7 kb from the left border (LB). Hybridising *nptII*-
specific fragments are flanked on one end by this internal
*Bgl*II site and on the other end by a genomic *Bgl*II site in the
region flanking the LB of the T-DNA. This will give rise to
25   hybridising bands of different length, dependent on the place
of integration, and can be used to determine the number of T-
DNA insertions in each transgenic plant.

The result of the southern analysis is shown in Figure 4.
30   Plant number 3028 (lane 3) is a non-transgenic escape from the
tissue culturing. All other plants are transgenic and
hybridise with the *nptII* probe. The copy number in the
transgenic plants varies from 1 to 4.

35   **5.1 Southern analysis of plants transformed with plasmid
pBBC10**

The copy number and the quality of the T-DNA insertion in transgenic plants obtained after transformation with plasmid pBBC10 (100 numbers) was determined by southern hybridisation. Aliquots of 5µg genomic DNA were digested for 16h with both

5   EcoRI and NcoI, separated on a 0.7% TAE agarose gel and transferred to a Hybond N+ membrane as described in 5.0.

The blot was probed with a 900bp ^{32}P radiolabeled C1-specific restriction fragment, obtained after digestion of plasmid

10   pFLAP10 with NcoI/PstI. Hybridisation and washing conditions were as described in section 5.0.

EcoRI cuts in the T-DNA immediately upstream of the P35s-c1-Tnos gene-fusion, 2.8 kb from the right border (RB).

15   Hybridising C1-specific fragments are flanked on one end by this internal EcoRI site and on the other end by a genomic EcoRI site in the region flanking the RB of the T-DNA. This will give rise to hybridising bands of different length, dependent on the place of integration, and can be used to

20   determine the number of T-DNA insertions in each transgenic plant.

NcoI cuts twice in the T-DNA region of plasmid pBBC10, at position +92 relative to the c1 translation startsite and in

25   the linker between the C1 gene and the nos terminator. Digestion of chromosomal DNA with NcoI therefore will give rise to a hybridising band of 1.5 kb.

The result of the southern analysis is shown in Figure 5 Plant

30   number 5002 is a transgenic plant containing a Pe8-gusA-Tnos insert and is used as a negative control. All other plants are transgenic and hybridise with the c1 probe. The copy number in these transgenic plants varies from 1 to 4 (panel A). After digestion with NcoI, all transgenic plants, except

35   5002, give a band of 1.5 kb, which runs at the same position

as the *NcoI*-digested pBBC10 plasmid (panel B). This indicates that all tested pBBC10 transformants contain at least one intact copy of the *C1* gene.

5   **5.2 Southern analysis of plants transformed with plasmids pBBC20 and pBBC30**

The copy number and the quality of the T-DNA insertion in transgenic plants obtained after transformation with plasmid pBBC20 (200 numbers) and pBBC30 (300 numbers) was determined
10   by southern hybridisation. Aliquots of 5μg genomic DNA were digested for 16h with *BglII/ClaI*, separated on a 0.7% TAE agarose gel and transferred to a Hybond N+ membrane as described in section 5.0.

15   To determine the copy number, the blot was probed with a 700bp ^{32}P radiolabeled *nptII*-specific fragment (see section 5.0). To determine the quality of the T-DNA insertion the *nptII*-probe was stripped from the blot according to the manufacturer's protocol (Amersham) and reprobed with a 0.7 kb
20   *Lc*-specific restriction fragment, obtained after digestion of plasmid pFLAP30 with *PstI/NcoI*. Hybridisation and washing conditions were as described in section 5.0.

BglII and *ClaI* in total cut four times in the T-DNA region of
25   pBBC20 and pBBC30 transformants. Relative to the left border, *BglII* is the first restriction site within the T-DNA. It cuts immediately upstream of the *nptII* gene, 1.7 kb from the left border (LB). Hybridising *nptII*-specific fragments are flanked on one end by this internal *BglII* site and on the other end by
30   a genomic *BglII* site in the region flanking the LB of the T-DNA. This will give rise to hybridising bands of different length, dependent on the place of integration, and can be used to determine the number of T-DNA insertions in each transgenic plant.

35

*Bgl*II cuts once in the *Pe8-lc-Trbc* gene-fusion of plasmids pBBC20 and pBBC30, at position +202 relative to the *lc* translation startsite. *Cla*I cuts immediately downstream of the *rbcS* terminator. Digestion of chromosomal DNA with *Bgl*II/*Cla*I therefore will give rise to a hybridising band of 2.6 kb with the *lc*-specific probe.

The results of the southern analyses are shown in Figure 6(*nptII* probe) and Figure 7 (*lc* probe). Plant number 004 is a non-transgenic control plant, which does not hybridise with any of the probes used. Plant numbers 5002, 104, 109 and 117 only hybridise with the *npt*II probe, and not with the *lc* probe. All other plants (200 and 300 numbers) are transgenic and hybridise with both the *npt*II and the *lc* probes. The copy number in these plants varies from 1 to 7 (Figure 6). After hybridisation with the *lc* probe, all 200 and 300 numbered plants give a band of 1.5 kb, which runs at the same position as the *Bgl*II/*Cla*I-digested pBBC20 and pBBC30 plasmids (Figure 7). This indicates that all tested pBBC20 and pBBC30 transformants contain at least one intact copy of the *Lc* gene.

Example 6: Measurement of flavonoids in tomato fruits

6.1   Growth and harvest of tomato fruits

Tomato plants were grown in 10 1 pots in a greenhouse at standard growth conditions. Fruits were harvested at fully red, ripe stage; and in the case of development studies (Figure 14) also at earlier stages of development. For discrimination between flavonoids in peel and in flesh tissue, the outer layer of about 2mm thick (i.e.cuticula, epidermal layer plus some sub-epidermal tissue) was separated from the fruit using a scalpel; the remainder of the fruit was classified as flesh tissue. After separation, tissues were quickly cut into pieces, frozen in liquid nitrogen and stored at -80°C until use. For analysis of flavonoid levels in whole fruits, red

fruits were cut into quarters, per fruit a quarter was taken, cut into pieces and quickly frozen.

6.2   Extraction of flavonoids from tomato tissues

5   Flavonoids were determined as aglycons or as their glycosides by preparing hydrolysed and non-hydrolysed extracts, respectively.

Preparation of hydrolysed extracts was performed according to
10   Hertog et al. (1992) as follows: Frozen tissues were grounded into a fine powder using either a pestle and mortar or a coffee grinder.   Peel and flesh tissues were lyophilized for 24h before flavonoid extraction.   Forty mg of this freeze-dried material was weighed and transferred to a 10ml Pyrex glass
15   tube.   To each tube 1.6ml of 62.5% methanol (HPLC grade) in distilled water and 0.4ml of 6M HCl were added.   The tubes were closed with screw caps containing a teflon inlay and incubated for 60 min at 90°C in a waterbath.   After hydrolysis, the tubes were cooled on ice, the extracts were diluted with 2ml of 100%
20   methanol and sonicated for 5 min.   For determination of aglycon levels in whole tomatoes, 1.2g of grounded frozen tissue (not lyophilized) was weighed and hydrolysed with 2ml of 100% methanol and 0.8ml of 6M HCl, as described above.   After hydrolysis, these whole tomato extracts were diluted with 4ml
25   of 100% methanol, and sonicated for 5 min.

Using flavonoid standards (obtained from Apin Chemicals Ltd, Abingdon, UK) it was established that during the hydrolysis step, aglycons were released from their respective glycosides
30   for 100%, while narichalcone (= naringenin-chalcone) was chemically converted into naringenin for more than 95%. Recoveries of quercetin, kaempferol and naringenin standards added to peel or flesh extracts just before hydrolysis were more than 90%.

35

For   determination   of   flavonoid-glycosides   and   naringenin-

chalcone, 40mg of freeze-dried tomato tissue was added to 4ml of 75% aqueous methanol acidified with HCl to pH2. Extraction took place at room temperature (20-25°C) by continuously mixing on a roller band for 2 hours.

5

6.3 HPLC conditions for flavonoid analysis

After sonication, about 1ml of each extract was taken using a disposable syringe and filtered through a 0.2µm PTFE disposable filter (Inacom Instruments BV, The Netherlands) before
10   injection into the HPLC system.

The HPLC system consisted of a Waters 600E Multisolvent Delivery System (Waters Chromatography), a Promis autoinjector (Separations Analytical Instruments BV) with a fixed 10µl loop,
15   and a Nova-Pak C_{18} (3.9 x 150mm, particle size 4µm) analytical column (Waters Chromatography) protected by a Guard-Pak Nova-Pak C18 insert. Both columns were placed in a LKB 2155 HPLC column oven (Pharmacia Biotech) set at 30°C. A photodiode array detector (Waters 996) was used to record spectra of
20   compounds eluting from the column on-line. The detector was set at recording absorbance spectra from 240 to 600nm with a resolution of 4.8nm, at a time interval of 1 second. Millennium 2010 Chromatography Manager (Waters Chromatography BV) was used to control the solvent delivery system and the
25   photodiode array detector.

HPLC separation of flavonoids present in hydrolysed extracts (flavonol aglycons and naringenin) was carried out under isocratic conditions of 25% acetonitril (for HPLC far UV) in
30   0.1% trifluoroacetic acid (TFA) at a flow rate of 0.9ml/min. HPLC separation of flavonoids in non-hydrolysed extracts (flavonoid-glycosides and naringenin-chalcone) was performed using a gradient of acetonitril in 0.1% TFA, at a flow rate of 1.0ml/min: 5-25% linear in 30 min, then 25-30% in 5 min and 30-
35   50% in 2 min followed by a 3 min washing with 50% acetonitril in 0.1% TFA. After washing, the eluent composition was brought

to the initial condition in 2 min, and the column was equilibrated for 6 min before next injection. Table 2 summarizes the retention times, obtained with the two different HPLC separation methods, of some flavonoid standards.

5

Table 2:   **Typical retention times of some flavonoid standards.**

		retention time (minutes)	
flavonoid standard	detection wavelength (nm)	isocratic run	gradient run
rutin	360	–	19.2
quercetin-3-glucoside	360	–	19.7
kaempferol-3-rutinoside	360	–	21.9
myricitin	370	3.0	23.0
quercetin	370	5.1	29.5
naringenin	280	8.3	33.5
narichalcone	360	–	34.8
kaempferol	370	9.8	35.5

Note: anthocyanins peaks would normally be visible in spectra
10   at 520 or 280 nm. In the experiments however these peaks are not visible (see e.g. Figure 25) meaning that no detectable levels of anthocyanins were formed in the tissues tested.

HPLC data were analyzed using the software of the Millennium
15   2010 Chromatography Manager. Absorbance spectra (corrected for baseline spectrum) and retention times of eluting peaks (with peak purity better than purity threshold value) were compared with those of commercially available flavonoid standards. Dose-response curves of quercetin, naringenin and kaempferol (0
20   to 20µg/ml) were established to quantify these compounds in the

hydrolysed tomato extracts. Quercetin and kaempferol aglycons were detected and calculated from their absorbance at 370nm and naringenin at 280nm. Flavonol-glycosides as well as naringenin-chalcone were detected at 360nm (see also Table 2).

5   Flavonoid levels in tomatoes were calculated either on a dry weight basis (for peel and flesh tissues) or on a fresh weight basis (for whole tomatoes). With the HPLC system and software used, the lowest detection limit for flavonoids in tomato extracts was about 0.1µg/ml, corresponding with 10mg/kg dry

10   weight and 1mg/kg fresh weight. Variation between replicate injections was less than 5%.

Example 7: **Characterisation of the flavonoid content in transgenic tomato fruit**

15

7.1 Flavonoids in peel and flesh of control tomatoes

The HPLC data of hydrolysed extracts of red fruit of variety FM6203 are shown in Figure 8. Figure 8a shows that both quercetin and kaempferol were present in peel tissue. In

20   contrast, Figure 8b illustrates that hydrolysed extracts of the flesh tissue from this fruit contained only traces of quercetin with no detectable levels of kaempferol. Chromatograms obtained at 280nm (not shown) of the same extracts revealed a large peak of naringenin in the peel, but not in the flesh.

25

Figure 9 shows the HPLC spectrum of non-hydrolysed extracts of this fruit. It can be seen that only a small peak corresponding to rutin was present in the flesh (Figure 9B). In contrast, at least 5 different flavonol-glycosides as well

30   as narichalcone were detected in the peel (Figure 9A). NMR-studies proved that the peak at RT = 19.2 min was rutin while the peak at 17.6 min was a quercetin-3-trisaccharide: rutin with apiose linked to the glucose of the rutinoside. The retention time and absorbance spectrum of the minor peak at

35   19.7 min corresponded with those of quercetin-3-glucoside, while those of the peak at 21.9 min corresponded with

kaempferol-3-rutinoside. The small peak at 25.6 min had an absorbance spectrum comparable to kaempferol-3-rutinoside, but its higher RT value indicates a yet unknown kaempferol-glycoside. The large peak at 34.8 min was narichalcone.

5   Aglycons of quercetin and kaempferol, as well as naringenin (all present in hydrolysed peel extracts) were not detectable in any of the non-hydrolysed extracts.

By comparing the flavonoid species in hydrolysed extracts with

10   those in non-hydrolysed extracts of the same tissue it can be concluded that the presence of quercetin and kaempferol aglycons in the hydrolysed extracts resulted from hydrolysis of their respective glycosides; the presence of naringenin in hydrolysed peel extracts resulted from isomerization of

15   narichalcone during the hydrolysis step (cf. Example 6.2).

These HPLC-data of flavonoids indicate that the flavonoid biosynthetic pathway is active in the peel but not, or to a very limited level only, in the flesh of the tomato fruit.

20   This conclusion was confirmed by mRNA analyses of peel and flesh tissues using probes for *chs*, *chi* and *fls*.

7.2   Flavonoids in fruits of transformed tomato plants

To determine whether the pBBC200 and pBBC300 constructs were

25   able to induce the flavonoid biosynthetic pathway in tomato plants, transformants as well as control plants were analysed for the presence of flavonoids in the flesh of their fruits, a tissue that normally contains only traces of flavonoids (see 7.1). This screening was performed by HPLC using hydrolysed

30   extracts. 17 plants that were successfully transformed with either the pBBC200 or the pBBC300 construct (i.e. *nptII*-positive plants) were analysed together with 10 untransformed control plants (escapes, i.e. plants that appeared *nptII*-negative after the transformation and kanamycin selection

35   steps). All untransformed control plants tested contained only a small amount of quercetin in hydrolysed extracts of the

tomato flesh and none had detectable levels of kaempferol or naringenin, as was the case with the original FM6203 plants. Out of the 17 transformants tested, 11 plants (65%) appeared to accumulate flavonoids, specifically of the kaempferol and

5   naringenin-type, at detectable levels in their tomato flesh. Figure 10 shows an example of chromatograms (recorded at 370nm) obtained with an untransformed control plant and plant number 3031 containing the pBBC300 gene construct. While the hydrolysed flesh extracts of the untransformed tomato contained

10   only a small peak of quercetin (cf. Example 7.1, Figure 8), the hydrolysed flesh extract of the transformed tomato contained a clear peak of kaempferol (Figure 10), as well as of naringenin (detected at 280nm, not shown). Figure 11 summarises the levels (on a dry weight basis) of kaempferol, quercetin and

15   naringenin, based on hydrolysed extracts, in the flesh of red fruits from some control plants and some plants transformed with either the pBBC200 or the pBBC300 gene construct. The main flavonoid present in the hydrolysed extracts of flesh of transformed plants was kaempferol, followed by naringenin; an

20   exception was plant number 3035, whose hydrolysed flesh extract contained more naringenin than kaempferol. Quercetin was not or only slightly increased in these transformants. Also in hydrolysed extracts of peel tissue of the pBBC200 and pBBC300 transformed tomato plants an increase in kaempferol content was

25   observed.

Figures 12 and 29 show the levels (on a fresh weight basis) of flavonoids in whole red fruits of control and transformed tomato plants. The level of total flavonols (quercetin plus

30   kaempferol) in fruits of control plants (first bar) was 5.2 ± 4.3 mg/kg fresh weight (mean ± standard deviation, n = 10), with values ranging between 1.3 and 12.0 depending on growing season. Quercetin was always the main flavonol in the fruits of control plants. Compared to the mean level in control

35   plants, 11 out of 13 pBBC200-transformed plants (i.e. 85%) and 11 out of 17 pBBC300-transformed plants (65%) had

significantly increased levels, up to 75 mg/kg i.e. 15 fold
increase, of total flavonols in their fruits. The highest
levels of kaempferol in transformants were about 75 mg/kg: a
75 fold increase compared to control levels. In contrast to
5   kaempferol, quercetin was increased in only a few plants and
to a much lower extent: up to 3 times the level in controls.
The level of naringenin in control fruits, derived only from
narichalcone present in the peel, was 24 ± 14 mg/kg fresh
weight (mean ± standard deviation, n = 10). The naringenin
10 level in whole fruits of transformants, derived from both
narichalcone and naringenin-glycosides (cf. Figures 27 and 25)
was significantly increased in a few plants: up to 2.5 fold
the level of control fruits.

15 Using non-hydrolysed extracts, the form in which the
flavonoids accumulated in the tomato fruits of transformed
plants was studied. By comparing HPLC chromatograms of non-
hydrolysed extracts from whole red fruits of control and
transformed tomatoes, recorded at 360 nm (Figure 26), it
20 appeared that the level of naringenin chalcone (peak at RT =
35.9 min was decreased in transformed tomatoes. However, at
least 6 other flavonoids detectable at 360 nm were increased
in the transformed tomato fruits: peaks at RT = 13.9, 14.3,
16.1, 22.8, 23.4 and 26.0 min . Apart from these 6 compounds,
25 the HPLC chromatograms recorded at 280 nm (Figure 27)
indicated an increase of at least another 5 flavonoid
compounds in the transformed tomatoes: peaks at RT = 11.2,
15.2, 21.2, 23.1 and 28.5 min. Other peaks that were
increased in the chromatograms of transformed fruits (e.g. in
30 Figure 14: peaks at RT = 5.1 and 11.9 min) appeared to be
impure peaks resulting of co-elution of yet unknown compounds.
Based on the chromatographic behaviour and absorbance spectra
of pure peaks that were increased in the transgenic fruits
(Figure 25), it is concluded that the compound eluting at RT =
35 22.8 min is kaempferol-3-O-rutinoside and that at 23.1 min is
naringenin-7-O-rhamnoglucoside (= naringin). The absorbance

spectra of the compounds eluting at 13.9, 14.3, 16.1, 23.4 and
26.0 min all match very well with kaempferol-3-rutinoside, but
their different RT values indicate that they are different
kaempferol-glycosides. Likewise, the compounds at RT = 11.2,
5   15.2, 21.2 and 28.5 min have absorbance spectra similar to
naringin, but their different RT values indicate that they are
different naringenin-glycosides.

The patterns of chromatograms (recorded at 360nm) obtained
10   with non-hydrolysed tomato extracts were very similar within
and between pBBC200 and pBBC300 transformants. This result
indicates that all transformants accumulated the same
flavonoid species at about similar ratios; transformants only
differed in their level of accumulation.
15
Figure 13 shows an example of chromatograms, recorded at 360
nm, of non-hydrolyzed extracts prepared from only the flesh
tissue of the tomato fruits (peel removed). While only rutin
was detectable in the flesh of control fruits, rutin as well
20   as the kaempferol-glycosides described above (Figure 26) were
detectable in the flesh of transgenic fruits. HPLC analyses
of the same flesh extracts at 280 nm (not shown) revealed
that, apart from these kaempferol-glycosides, the naringenin-
glycosides described above (Figure 27) were exclusively
25   produced in transgenic fruits. These results indicate that
the flavonoids biosynthetic pathway in transgenic plants is
not restricted to the peel, as it is in untransformed plants,
but also active in the flesh of the fruits.

30   Out of the 11 flavonoid species that were increased in the
whole fruits of transgenic plants (Figure 26) only kaempferol-
3-rutinoside was also found in the fruits of FM6203 control
plants, though only in the peel. Thus, tomato plants
transformed with pBBC200 or pBBC300 exclusively produced (at
35   least) 6 different kaempferol-glycosides and 5 naringenin-
glycosides in the flesh of their fruits. Moreover, except for

kaempferol-3-rutinoside, the flavonoid species induced by pBBC200 or pBBC300 are new products in the fruits of variety FM6203.

5   The accumulation of flavonoids in fruits of transformed plants was dependent upon ripening stage. Figure 14 shows that the kaempferol peak was not detectable in hydrolysed extracts of flesh from full-grown tomato fruits at green stage, small at turning stage and highest at red stage. A similar ripening-
10   dependent increase was observed for naringenin (chromatograms recorded at 280 nm; not shown). This timing of the flavonoid biosynthesis correlated with the pattern of activation of the e8 promotor, which is present in both the pBBC200 and the pBBC300 gene construct.
15

In some plants transformed with pBBC200 or pBBC300 a slight purple pigmentation due to anthocyanin accumulation was observed in the fruits, but only at green and breaker stage. Binocular analyses of cross sections of these purple-coloured
20   fruits revealed that the anthocyanin accumulation was restricted to the peel tissue of the fruits. However, anthocyanins could not be detected, neither by eye nor by HPLC analyses of hydrolysed and non-hydrolysed extracts at 520 nm, in red fruits. In contrast to this pattern of anthocyanin
25   production, the production of kaempferol- and naringenin-glycosides in the same transgenic fruits was not restricted to the fruit peel and increased during fruit ripening (see above). Therefore, the induced biosynthesis of flavonoids in tomato fruits, as claimed in the present invention, is not
30   related to the production of anthocyanins.

7.3   **Transformation with both Lc and C1 is essential for increasing flavonoid levels in tomato fruits**

To check whether C1 or Lc alone might as well induce the
35   flavonoid biosynthesis in tomato fruits, variety FM6203 was also transformed with the plasmids pBBC10, pBBC20 and pBBC30

containing the single regulatory gene constructs *d35s-cl*, *e8-lc* minus leader sequence and *e8-lc* plus leader sequence, respectively. Hydrolysed extracts were prepared from whole red fruits and analysed by HPLC as described in Example 6.

5   Figure 28 shows that fruits of plants transformed with only *Cl* (plants numbered 101 onwards) or only *Lc* (plants numbered 201 and 301 onwards for plants without and with leader sequence, respectively), had levels of total flavonols (quercetin plus kaempferol) ranging from 1.2 to 8 mg/kg fresh weight. i.e.

10   within the range of control fruits (1.8 to 8.8 mg/kg fresh weight). These data indicate that transformation of tomato plants with either *Cl* or *Lc* (with or without leader sequence) did not result in increased flavonol levels in their fruits. In contrast, fruits of plants containing both regulatory genes

15   after transformation with pBBC200 or pBBC300 (numbered 2000 and 3000 onwards, respectively) had significantly increased levels of flavonols in their fruits, as compared to both the control plants and the plants transformed with the single gene constructs. This increase in flavonols was mainly due to the

20   accumulation of kaempferol (Fig 29). Evidently, the presence of two transcription factor genes such as both *Cl* and *Lc* is essential for the increased production of flavonoids in tomato fruits.

25   Similarly the level of flavonols can be measured in the leaves of the transformed tomatoes. It was found that the the levels of flavonols in the leaves was increased up to six-fold as compared to the control and mostly were at a level lower than the flavonol level in the flesh of the fruit. In none of the

30   cases lethal dosis of flavonols were encountered, this may be explained by the fact that a fruit-specific promoter was used, which although it apparently does not fully prevent formation of flavonols in leaves it limits the levels to non-lethal dosis.

35

Example 8: Analysis   of   transgene   expression   in   transgenic

plants

The expression of the introduced *Cl* and *Lc* genes in the obtained transgenic tomato plants was analysed by real-time quantitative RT-PCR, using the fluorogenic 5' nuclease assay performed with the Taqman PCR reagent kit on the ABI PRISM 7700 sequence detection system (Perkin -Elmer/ABI). The principle of this procedure is as follows: cDNA is made from total RNA, extracted from the source of interest, by reverse transcription. The expression of a target gene is monitored in a PCR reaction to which a fluorogenic probe, consisting of an oligonucleotide with both a reporter and a quencher dye attached, is added, that anneals specifically between the forward and reverse primers. When the probe is cleaved by the 5' nuclease activity of the DNA polymerase, the reporter dye is separated from the quencher dye and a sequence-specific signal is generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored during the PCR reaction. The PCR cycle at which the fluorescence reaches a certain threshold value is a measure for the starting copy number of the target RNA. This can be quantified by running a standard range with known amounts of the target gene.

The following plants were selected for Taqman analysis: plants transformed with plasmid pBBC10 (*P35s-cl-Tnos*), numbers 104, 109 and 117 plants transformed with plasmid pBBC20 (*Pe8-lc⁻-Trbc*), numbers 201, 204 and 209 plants transformed with pBBC30 (*Pe8-lc⁺-Trbc*), numbers 302 and 305; plants transformed with pBBC300 (*P35s-cl-Tnos//Pe8-lc⁺-Trbc*), number 3031; and plants transformed with plasmid pBBC250 (*Pe8-cl-Tnos//Pe8-lc⁻-Trbc*), numbers 2509, 2511 and 2519. In plasmid pBBC250 both the *Cl* and the *Lc* gene are expressed under control of the fruit-specific tomato *e8* promoter. Plasmid pBBC250 is represented in Figure 20 and can be prepared analogously to plasmids pBBC200 and pBBC300, transformants with plasmid pBBC250 are

numbered 2500-2999.

Total RNA was isolated from red fruits of transgenic tomato
plants. First strand cDNA was synthesised from 350 ng of
5   total RNA by reverse transcription. Aliquots of 100 ng cDNA
were used in three Taqman PCR reactions with a *c1*, *lc* and a
cyp probe respectively. The tomato cyclophyllin gene (*cyp*) is
constitutively expressed in tomato fruits, as observed by
northern analysis, and can therefore be used as internal
10   control.   Known amounts of plasmid pFLAP300 and a 0.4 kb
cyclophyllin PCR fragment, obtained after RT-PCR of tomato
fruit RNA with primers CYP1S and CYP2A (Table 1), were used as
standards. The sequence of the taqman primers and probes used
is listed in Table 4.
15

For all three primer-probe combinations, the number of
specific mRNA molecules present in each sample was determined
and expressed relative to the amount of *cyp* mRNA.   The
absolute amount of *cyp* mRNA varied less than 2.5-fold between
20   the different transgenic plants, indicating that this gene can
indeed function as an internal control. As shown in Figure
21, control plant 004 neither gave a signal with the *c1* probe,
nor with the *lc* probe.   In contrast, all tested transgenic
plants showed a clear expression of the introduced transgenes:
25   the *C1* single-gene plants (104, 109 and 117) showed only *C1*
gene expression, the *Lc* single-gene plants (201, 204, 209, 302
and 305) only showed *Lc* gene expression and all tested *C1/Lc*
double-gene plants (2509, 2511, 2519 and 3031) showed
expression of both *C1* and *Lc*. Although the expression level
30   of the *C1* and *Lc* genes in some single-gene transgenic plants
is higher than the levels found in some double-gene plants,
yet there is no detectable increase in the level of kaempferol
(Figure 21). This indicates that expression of both *C1* and *Lc*
together is required to up-regulate the flavonoids pathway in
35   tomato fruits, leading to an increased production of the
flavonol kaempferol.

Table 4:   **Overview of Taqman primers and probes used**

Primer/probe(*)	gene	sequence (5' to 3')
C1F	C1	GCCCTGGCGTCGTTTCT
C1R	C1	TGGACATCTATACGTGTACTTGTTGTCTAC
C1T(*)	C1	CTCCGCTGTCAGACGGCCGG
LCF	Lc	CGGGAGCAGCACAGGAAAT
LCR	Lc	GTCGCTTTCGCTCCGACAT
LCT(*)	Lc	TGGCACTGGCACCAAGAACCACG
CYPF	cyp	GAGTGGCTCAACGGAAAGCA
CYPR	cyp	CCAACAGCCTCTGCCTTCTTA
CYPT(*)	cyp	ACATCCATGCCTTCAACAACTTGTCCAA

5   (*) Fluorescently labeled Taqman probes.

Example 9: **Tomato fruits with increased flavonoid levels by transformation with lc and c1 exhibit an increased antioxidant activity**

10

Because the postulated beneficial effects of flavonoids in the human diet is, at least partly, ascribed to their antioxidant characteristics, it was tested whether tomato plants with increased levels of flavonoids in their fruits also have

15   increased antioxidant activities.

From 3 control and 3 independent transformants [numbers 3031, 3059 and 3060 as described above], 0.6 g FW of whole fruits (grinded material from 3 red fruits pooled per plant) was

20   weighed, extracted in 2 ml of 70% methanol by sonication for 30 min, and filtered (0.2 μm). Antioxidant activity was tested from the ability of extracts to react with the coloured radical cation of ABTS (2,2'-azinobis[3-ethylbenzothiazoline-

6-sulphonic acid]), essentially as described in Miller and Rice-Evans (1997). The ABTS-radical stock solution, generated with potassium persulphate, was diluted in 5 mM potassium phosphate (pH 7.4) containing 150 mM NaCl just before use,

5 obtaining a working solution with an absorbance value of 1.240 measured at 734nm. The antioxidant activity of tomato extracts was assayed by adding 10 µl of extract to 990 µl of the ABTS-radical working solution, mixing for 30 sec and reading the absorbance at 734nm exactly 1 min after the

10 addition of extract. A calibration curve of trolox (0 – 20 nmol) was prepared to calculate the trolox-equivalent antioxidant capacity values (TEAC values) of the tomato extracts. Variation between replicates was always less than 7%.

15

The TEAC value of control tomato extracts was 2513 ± 409 µmol/kg FW (mean ± standard deviation of 3 plants). Compared to this control value, the TEAC values of the transgenic plants were increased by 20% in 3031, 40% in 3059 and 64% in

20 3060. Figure 22 shows that the TEAC values of the fruits were closely related to the levels of total flavonoids in these fruits, in both control and transgenic plants. This result suggests that the increased antioxidant activity observed in the fruits of *lc/c1*-transformed plants resulted from the

25 increased flavonoid biosynthesis.

Example 10:   **properties of tomato pastes prepared from transformed tomatoes of the invention**

30 This example demonstrates that overexpression of transcription factors in tomato can improve commercially-important properties such as paste consistency, in particular by leading to thicker pastes at the same fruit usage level.

35 Data from five independent paste preparations are included in this example. Pastes 1 and 2 are from T1 generation plants

(i.e. a population derived from seed of the selfed primary transgenic plant 2059). Pastes 3, 4 and 5 are derived from T2 homozygous plants.

10.1 Plant Material

10.1 Growing

Seed from the selfed single-insert 2059 primary transgenic tomato plants were sown in soil flats in a glasshouse under light conditions as in Example 1. Seedlings which had retained the pBBC300 transgene were selected by PCR, using the transgene specific primer pair F69/F72 (table 1). PCR positive transgenic plants were designated 2059+. PCR negative control plants having lost the pBBC300 transgene through segregation were designated 2059-. FM6203 (the parent line) was also planted as a control.

After selection by PCR, 16-20 day old seedlings were transferred to an automatic hydroponics feeding and watering system.

Seeds from homozygous selfed T1 plants were planted in soil flats in a glasshouse. 16-20 day old seedlings were transferred to an automatic hydroponics feeding and watering system.

Fruits were harvested between 18-21 days post breaker (breaker represents the stage were the first flush of orange colour appears on the developing fruit). Fruits were used for paste production within 2-4 hours of harvest.

10.2 Preparation of tomato paste

450-850 g of fruit was diced (15 mm) and microwaved in a Pyrex bowl for 4, 2 and 1 minutes (full power) stirring between each period of heating. The pulp was chilled to room temperature on

melting ice and H$_2$O added to bring the cooked tomato pulp upto the original wet weight. Chilled juice was sieved through meshes of 1400 and 710µm whereby losses (seed + skin) of around 10-14% were typically incurred.

5

A weighed amount of hot break juice was centrifuged at 5,000 RPM for 5 min, the serum decanted and the serum and pellet weights determined (serum/pellet ratio). The serum pellet ratio is a measure of particle occupancy — the thicker the
10   paste the lower the serum/pellet ratio. Paste prepared using tomatoes from the 2059+ line possessed consistently lower serum/pellet ratios than those prepared from either 2059— or parent FM6203 lines (Figure 23).

15   Pastes were reconstituted by blending back a portion of the serum (typically to 75% of juice weight) to the pellet, allowing a minimum of 1 hour at room temperature for the particles to re-swell.

20   Bostwick represents the Industry standard way for measuring paste viscosity. Approx. 100g of this 75% paste (prepared as above) was placed in the chamber of the levelled Bostwick, the shutter opened and the distance (in cm) over which the paste flows in 30 sec. was recorded. Pastes with a low Bostwick
25   value have a high viscosity and *vice versa*.

Pastes prepared with tomatoes derived from the 2059+ line possessed consistently lower Bostwick values than those prepared from the 2059— or parent FM6203 line (Figure 24).
30

These results show that tomato lines of the invention provide advantageous properties to tomato products, for example they can provide increase thickness of products such as tomato pastes.
35

Example 11:   **Transformation of tomato plants with *Lc* and *Cl***

increases the production of methylsalicylate
in the fruits

The effects of the regulatory genes lc and cl on the
5   production of volatiles produced by the tomato fruits were
tested, using solid phase microextraction (SPME) and gas
chromatography coupled to mass spectrometry (GC-MS).

In a 4 ml glass vial, 0.5 g of frozen material (fresh weight
10 basis) of grinded whole red fruits was weighed. After
thawing, the fruit material was incubated at room temperature
with gently stirring for 5 min, to allow enzymatic reactions.
The reaction was stopped by adding 0.37 g of solid $CaCl_2$ and
the vial was quickly closed with an open top crimp cap with
15 teflon inlay. The vial was placed in a waterbath at 50°C
above a stirring device. After 10 minutes of preincubation
while stirring, the volatiles in the headspace were sampled
during 15 minutes, using SPME with a silica fiber coated with
100 μm polydimethylsiloxane (Supelco Inc., Bellefonte, PA,
20 USA). Volatiles trapped were identified by gas chromatography
and mass spectrometry (GC-MS) according to Verhoeven et al.
(1997).

Figure 30 shows typical GC-MS chromatograms of volatiles
25 produced by red tomatoes of both untransformed and transgenic
FM6203 plants. Methylsalicylate production appeared
significantly higher in fruit extracts of plants expressing
both Lc and Cl: its peak area, expressed as total ion counts x
10^6, in control plants was 9.2 ± 1.6 (mean ± s.d.; n = 2),
30 while in plants independently transformed with both Lc and Cl
it ranged from 17.2 to 32.2 (mean ± s.d.: 29.6 ± 11.4; n = 3).
In contrast, methylsalicylate production was not changed in
fruits of plants expressing only lc (Figure 30B): in these
transgenic plants the mean ± s.d. of the peak area was 10.8 ±
35 2.1 (n = 2). Of the two tested plants expressing only cl in
their fruits, one had a level comparable to that in the

untransformed control plants, while the other (Figure 30C) produced methylsalicylate similar to plants expressing both *C1* and *Lc*. The applicants therefore speculate that the increase in the production of methylsalicylate in the fruits of plants

5   transformed with the regulatory genes *Lc* and *C1* is mainly due to the action of *C1*.

A significant increase in methylsalicylate level of tomatoes of the invention as compared to control fruits was found.

10   Methylsalicylate is a key pathogen wound response volatile signalling molecule in plants. Its biosynthesis via the phenylpropanoid pathway probably involves benzoic acid as a precursor.

15   Surpisingly the combined insertion of *Lc+C1* not only provide increased levels of flavonoids but also increased levels of Methylsalycilate and therewith offers possibilities for the selective tailoring of plants to produce altered levels of targeted aroma compunds and novel flavoured food materials.

20

CLAIMS

1. A method for producing a plant capable of exhibiting altered levels of flavonoids other than anthocyanins comprising incorporating into said plant two or more genes each encoding a different transcription factor for flavonoid biosynthesis, or a sequence functionally equivalent thereto, each gene being operably linked to a promoter.

2. A method according to claim 1, wherein the plant is an edible plant, preferably selected from the group of vegetables such as a tomato plant, spinach, a pea plant, broccoli, cauliflower, asparagus and potato plant, fruit-bearing plants such as a strawberry plant, oil producing plants such as sunflower, soybean and rape, or extractable plants such as tea plants.

3. A method according to claim 2 wherein the plant is a tomato plant.

4. A method according to any one of claims 1 to 3 wherein flavonoid levels other than anthocyanins in the plant are increased.

5. A method according to any one of claims 1 to 4 wherein the level of flavonoids other than anthocyanins in the flesh of the fruit is increased.

6. A method according to any one of claims 1 to 5 wherein the flavonoids other than anthocyanins include flavonols.

7. A method according to any one of claims 1 to 6 wherein the combination of genes comprises a gene encoding a *myb*-type transcription factor in combination with a gene encoding a *myc*-type transcription factor.

8.   A method according to claim 7 wherein the combination of
      genes comprises a gene encoding the maize *C1* transcription
      factor in combination with a gene encoding the maize *Lc*
5   transcription factor.

9.   A method according to any one of claims 1 to 8 wherein at
      least one of the genes encoding a transcription factor is
      operably linked to a non-constitutive promoter and/or to a
10   tissue-specific promoter.

10.   A method according to any one of claims 1 to 9 wherein the
      combination of genes comprises a gene encoding the maize
      C1 transcription factor, operably linked to the
15   constitutive double 35S cauliflower mosaic virus promoter
      in combination with a gene encoding the maize *Lc*
      transcription factor, operably linked to the fruit-
      specific tomato E8 promoter.

20 11. A plant having two or more transgenes each encoding a
      different transcription factor for flavonoid biosynthesis,
      or a sequence functionally equivalent thereto, stably
      incorporated into its genome such that its ability to
      produce flavonoids other than anthocyanins is altered.
25

12.   A plant according to claim 11 prepared according to the
      method of any one of claims 1 to 10.

13.   A DNA construct comprising sequences coding for a
30   combination of two or more genes, each gene encoding a
      different transcription factor for flavonoid biosynthesis,
      or a functionally equivalent sequence thereof, each gene
      being operably linked to a promoter.

35 14. A construct according to claim 13 comprising as operably
      linked components in an expression cassette two or more

units each comprising a promoter functional in a plant cell, a gene encoding a transcription factor for flavonoid biosynthesis and a transcriptional and translational termination region functional in a plant cell.

15.   A plant comprising a DNA construct according to claim 13 or 14.

16.   A transformed plant having flavonol levels compared to similar, untransformed plants.

17.   A tomato plant having enhanced flavonol levels in the flesh of the fruit.

18.   Seeds, fruits, progeny and hybrids of a plant according to any one of claims 11, 12, 15, 16, or 17.

19.   Use of a plant according to any one of claims 11, 12, 15, 16 or 17 in the preparation of a food product or a skin or hair protective product.

20.   A food product prepared by the method of claim 1, having enhanced flavonol levels.

21.   A food product according to claim 20, wherein the food product is a tomato product such as sauce, paste, dressing, ketchup or soup.

22.   A food product according to 21 having an improved flavour and/or an increased thickness.

23.   A method for producing a plant capable of exhibiting altered levels of flavour compounds or precursors thereof, such as methylsalicylate, comprising incorporating into said plant two or more genes each encoding a different transcription factor for flavonoid biosynthesis, or a sequence functionally equivalent thereto, each gene being

operably linked to a promoter.

24.   A method of increasing the thickness of tomato pastes, by using tomatoes as an ingredient thereof, said tomatoes being produced by a plant which has incorporated into its genome two or more genes each encoding a different transcription factor for flavonoid biosynthesis, or a sequence functionally equivalent thereto, each gene being operably linked to a promoter.

Fig.1.

Fig.1 (Cont).

Fig.2.

Fig.3(a).

Tg7　nptⅡ　Pnos　Pd35s　　c1　　Tnos

LB　　　　　　　　　　　　　　　　　　　　　　RB

Fig.3(b).

Tg7　nptⅡ　Pnos　　Pe8　　　　　Ic⁻　　Trbc

LB　　　　　　　　　　　　　　　　　　　　　　RB

Fig.3(c).

Tg7　nptⅡ　Pnos　　Pe8　　　　　Ic⁺　　Trbc

LB　　　　　　　　　　　　　　　　　　　　　　RB

Fig.3(d).

Tg7　nptⅡ　Pnos　Pd35s　c1　Tnos　　Pe8　　　Ic⁻　　Trbc

LB　　　　　　　　　　　　　　　　　　　　　　　　　　RB

Fig.3(e).

Tg7　nptⅡ　Pnos　Pd35s　c1　Tnos　　Pe8　　　Ic⁺　　Trbc

LB　　　　　　　　　　　　　　　　　　　　　　　　　　RB

Fig.4.

Fig.5(a).

Fig.5(b).

Fig.6.

Fig.7.

Fig.8(a).

Fig.8(b).

Fig.9(a).

Fig.9(b).

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Fig.10(a).

Fig.10(b).

Fig.11.

Fig.12.

Fig.13(a).

Fig.13(b).

Fig.14(a).

Fig.14(b).

Fig.14(c).
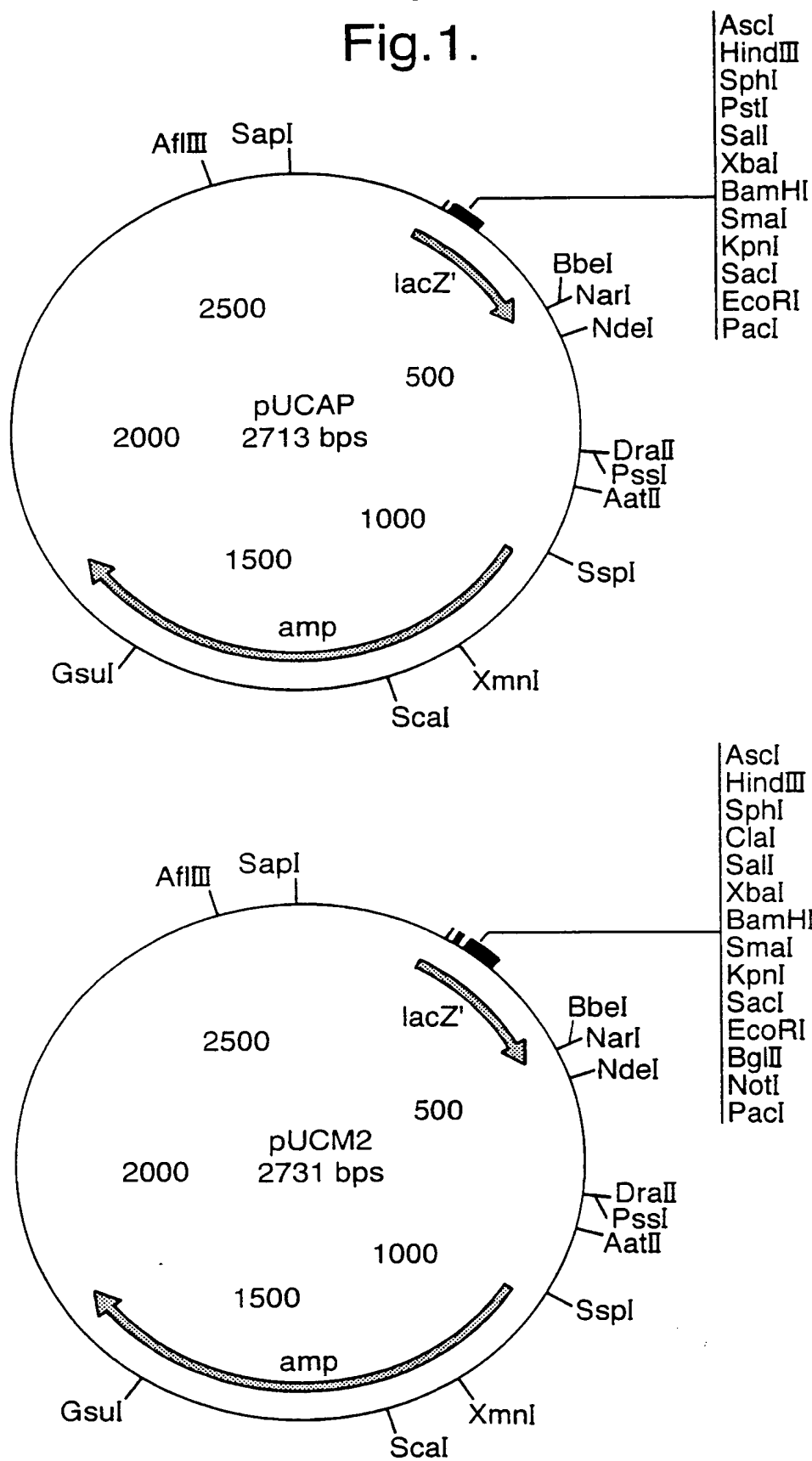
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Fig.15.
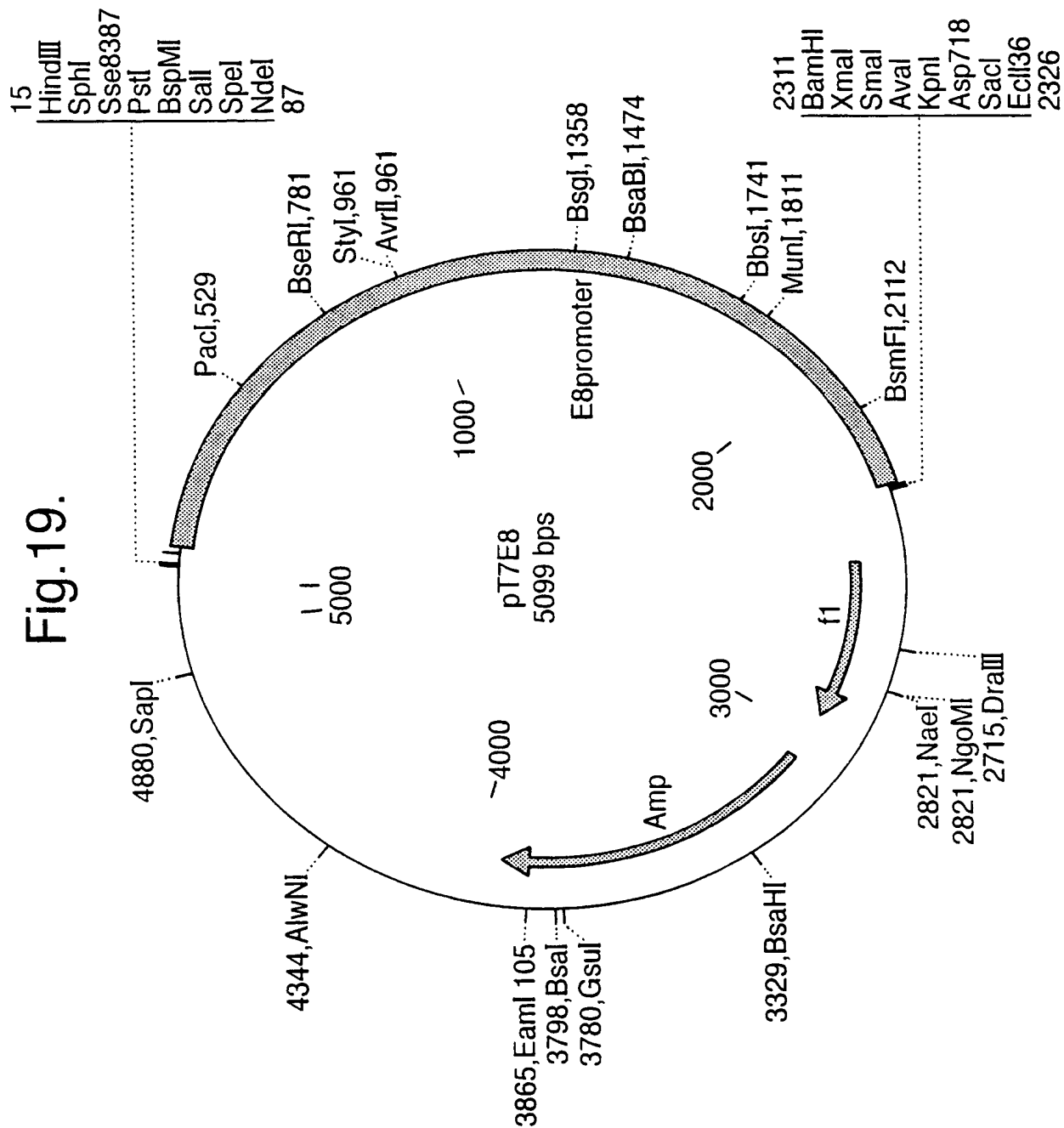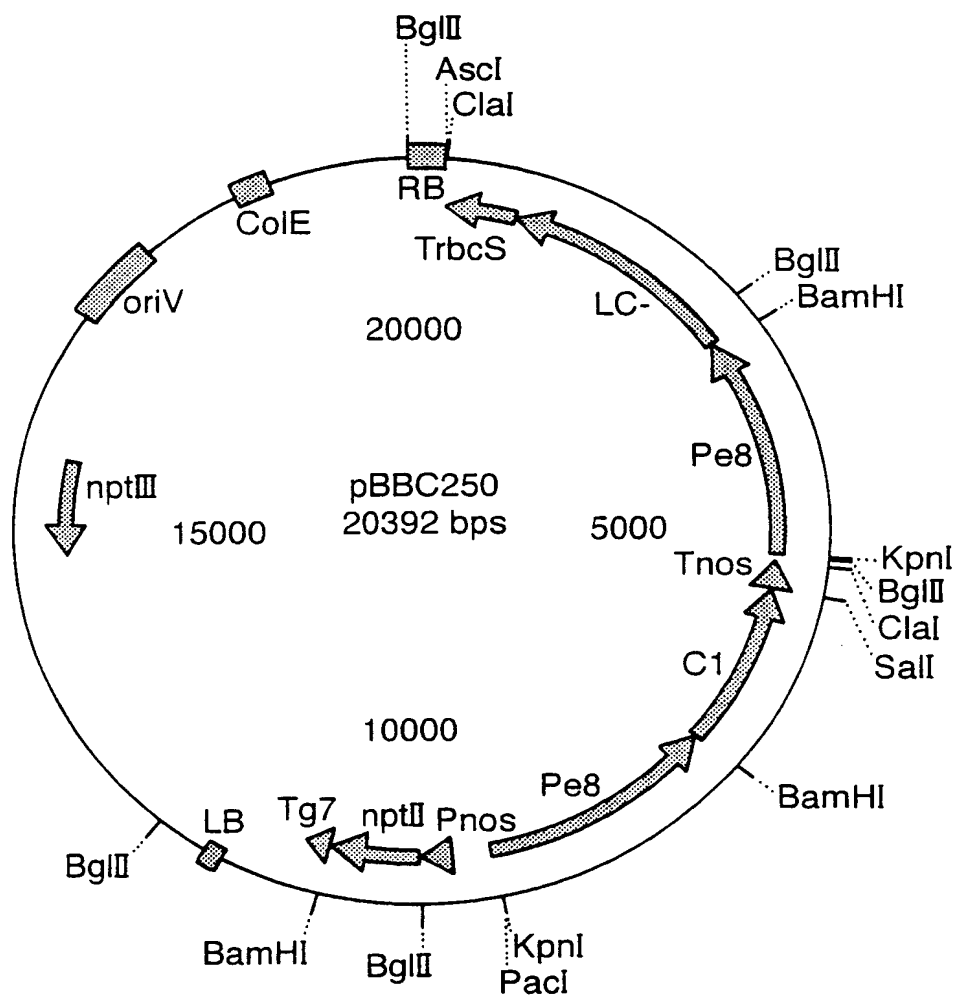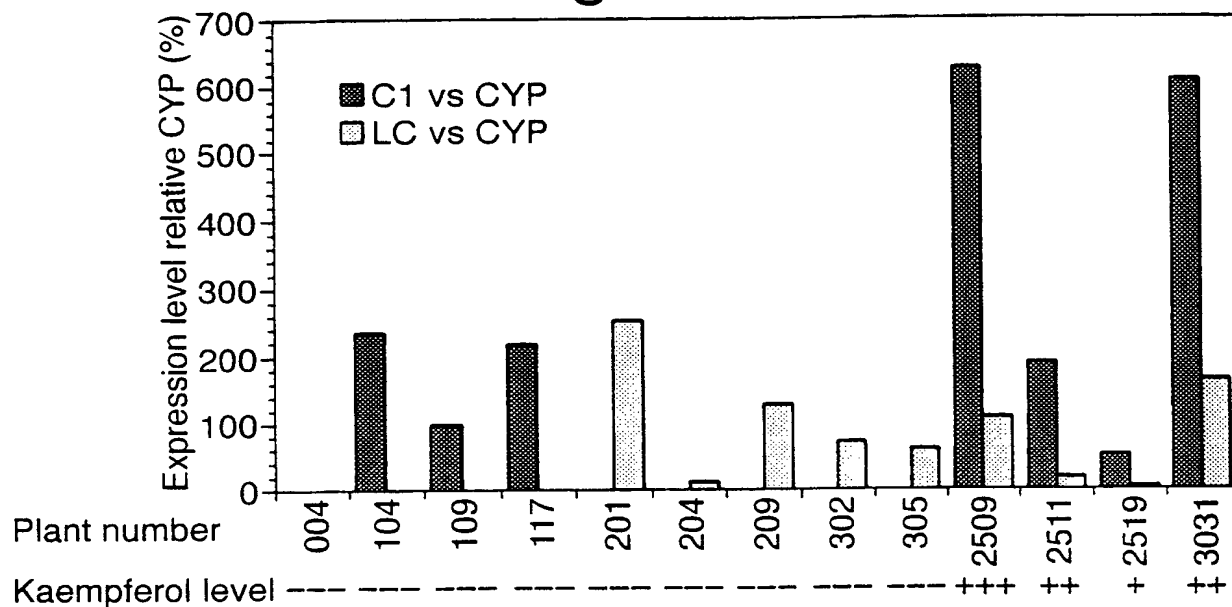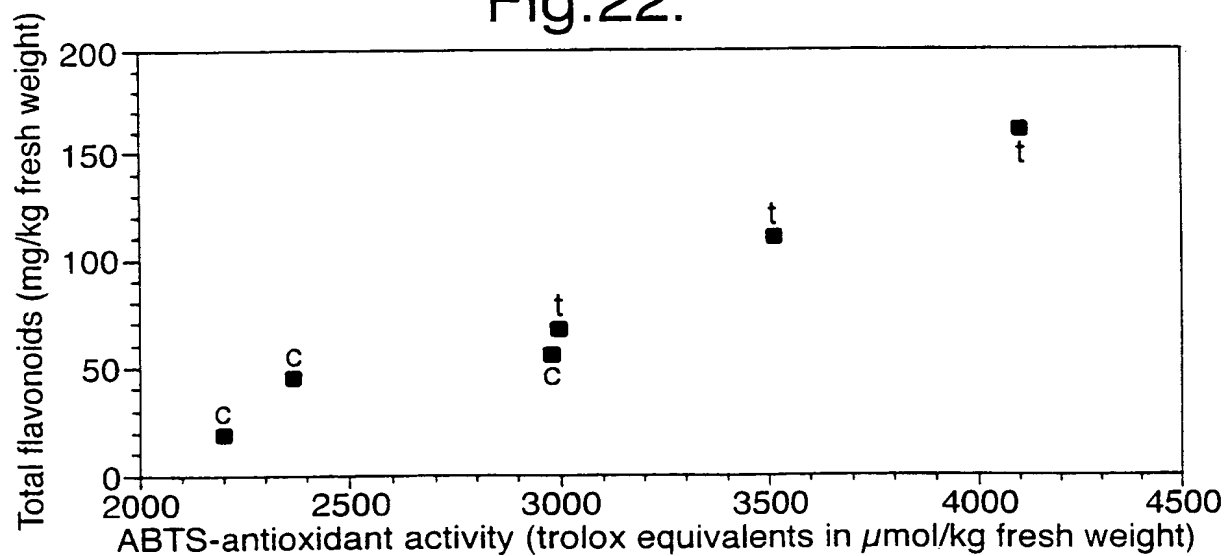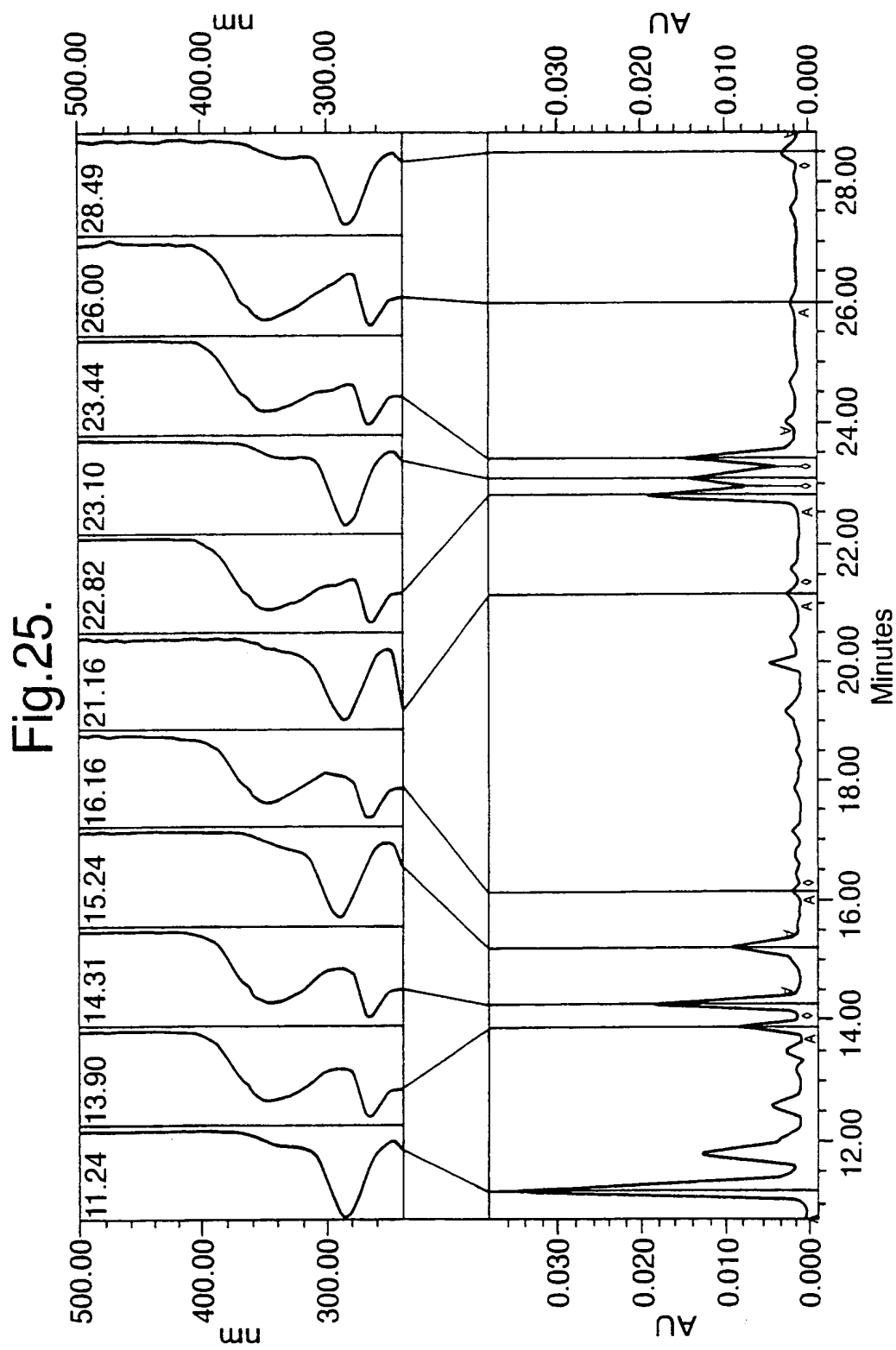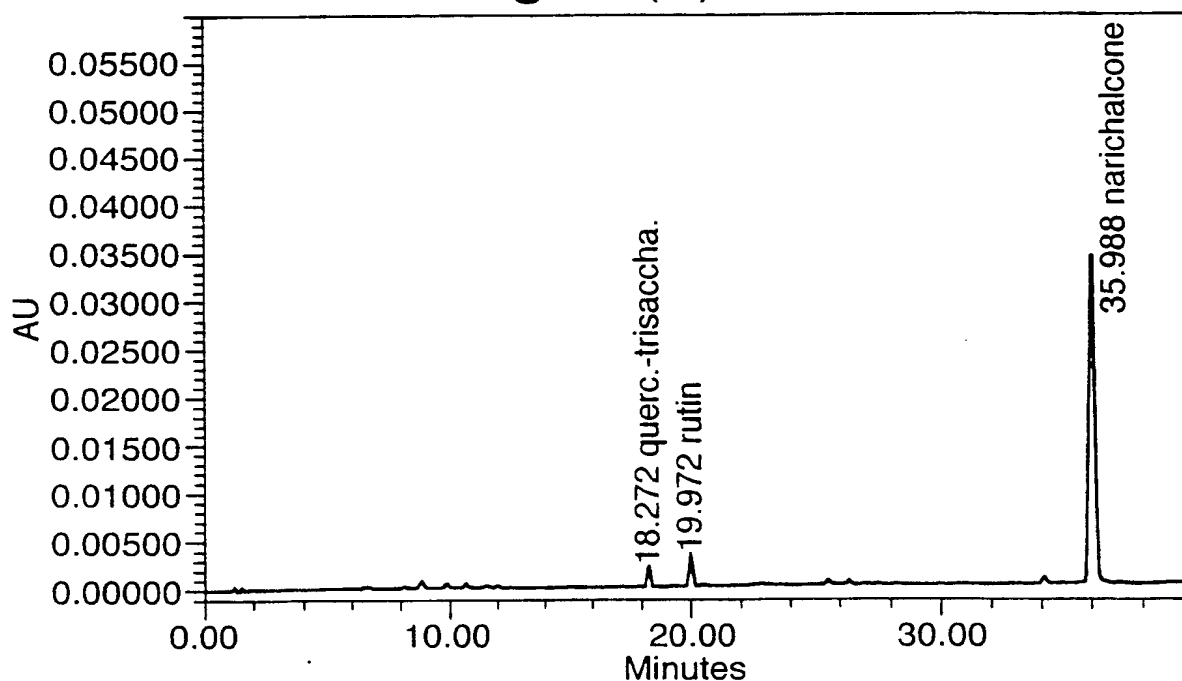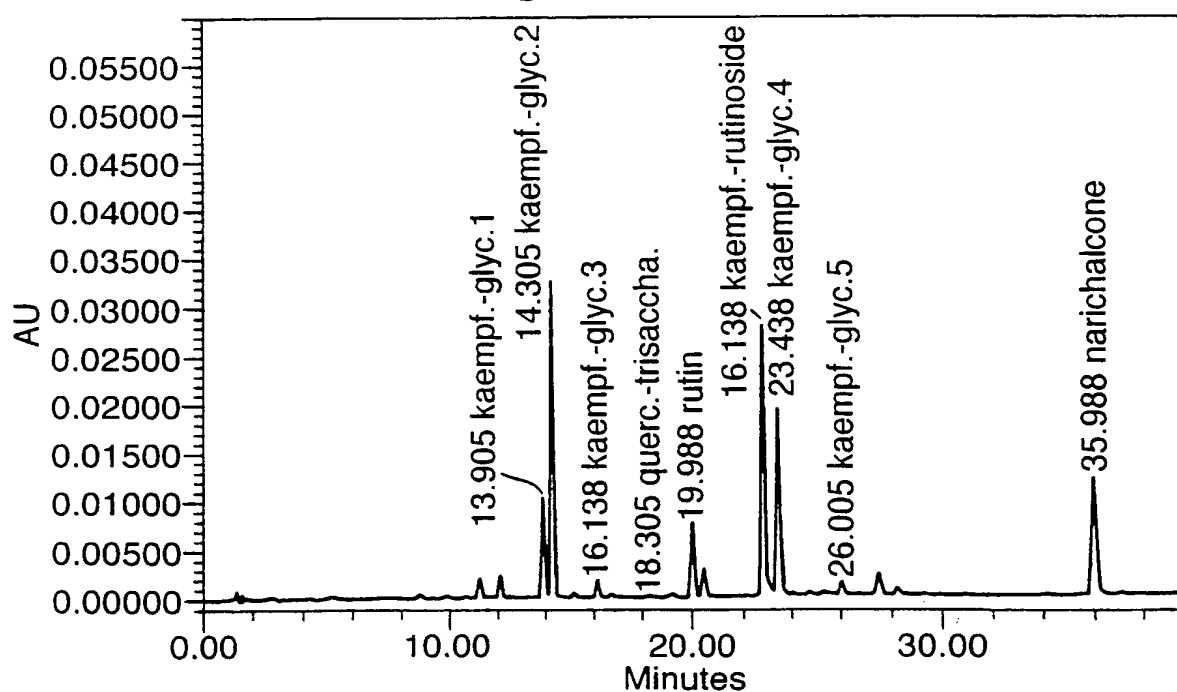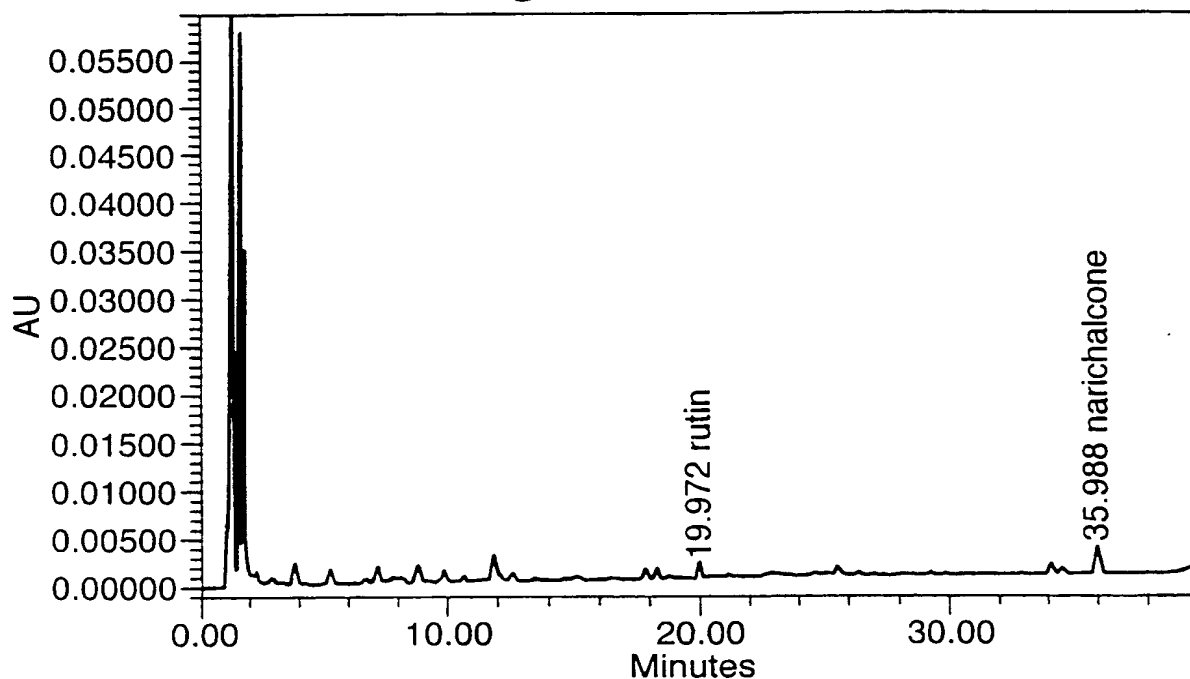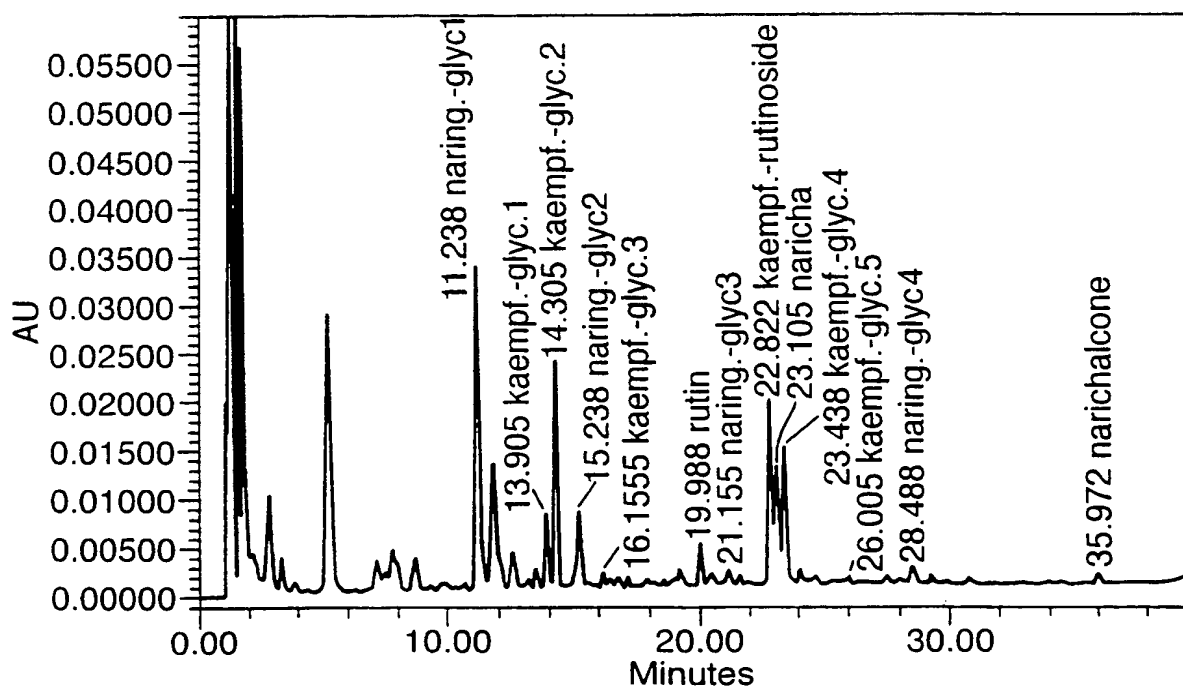
Fig.15 (Cont).

Fig.16.

Fig.17.

Fig.17 (Cont).

Fig.18.

Fig.19.

Fig.20.

Fig.21.

Fig.22.

Fig.25.

Fig.26(a).

Fig.26(b).

Fig.27(a).

Fig.27(b).

Fig.28.

Fig.29(a).

Fig.29(b).

Fig.29(c).

Fig.29(d).

Fig.30(a).

8000327

Fig.30(b).

6294179

Fig.30(c).

30271076

Fig.30(d).

32363180
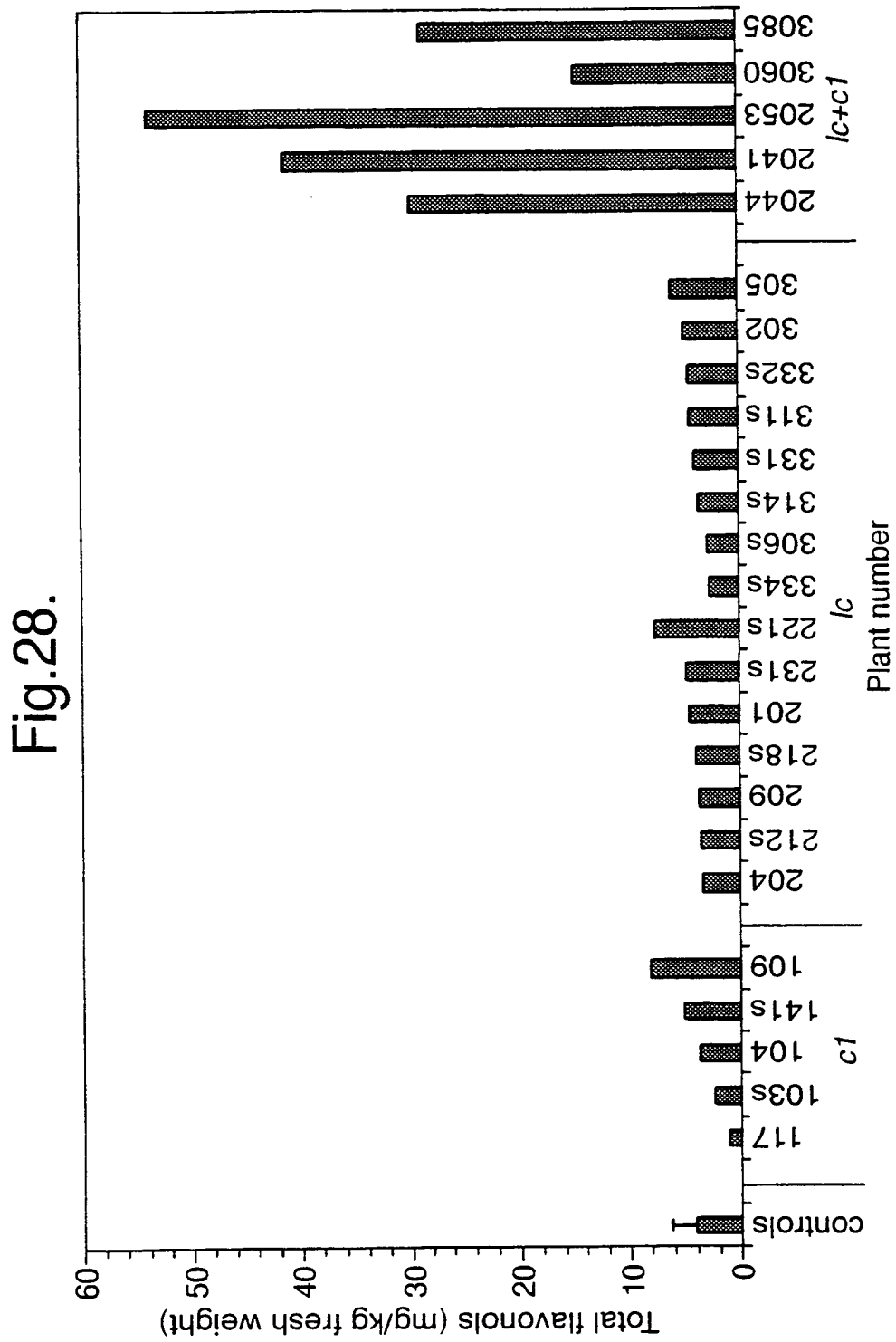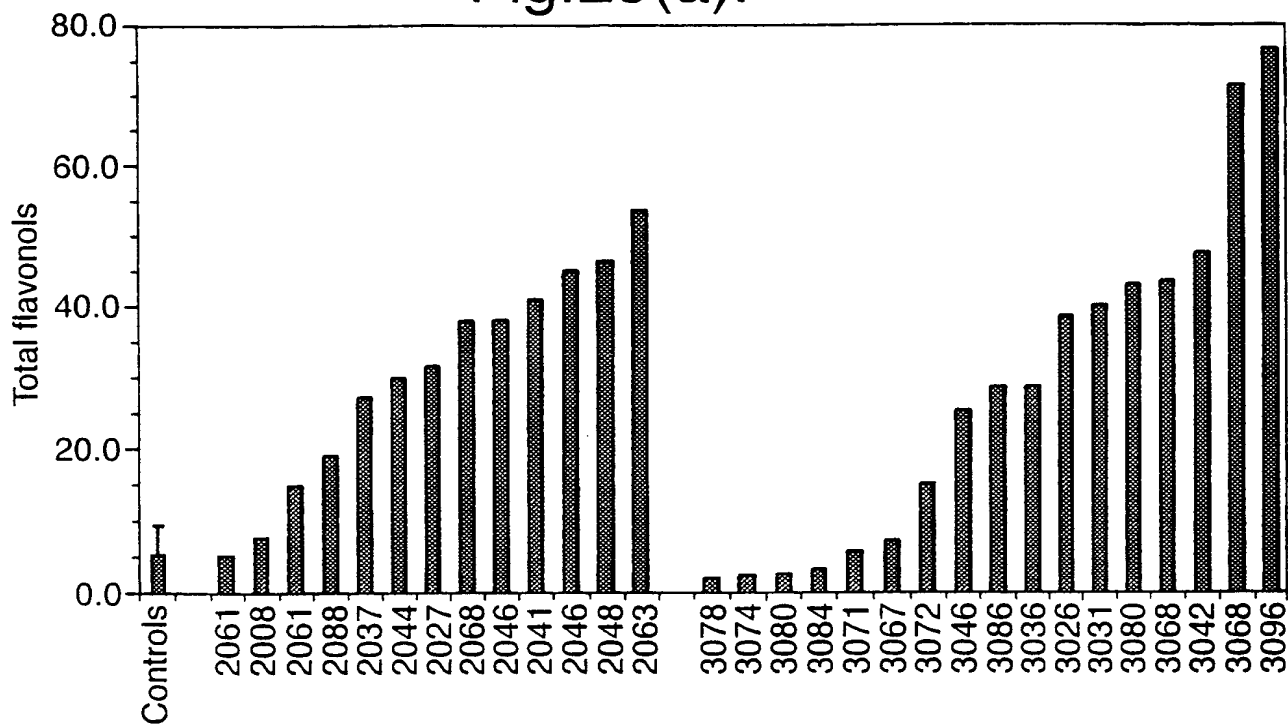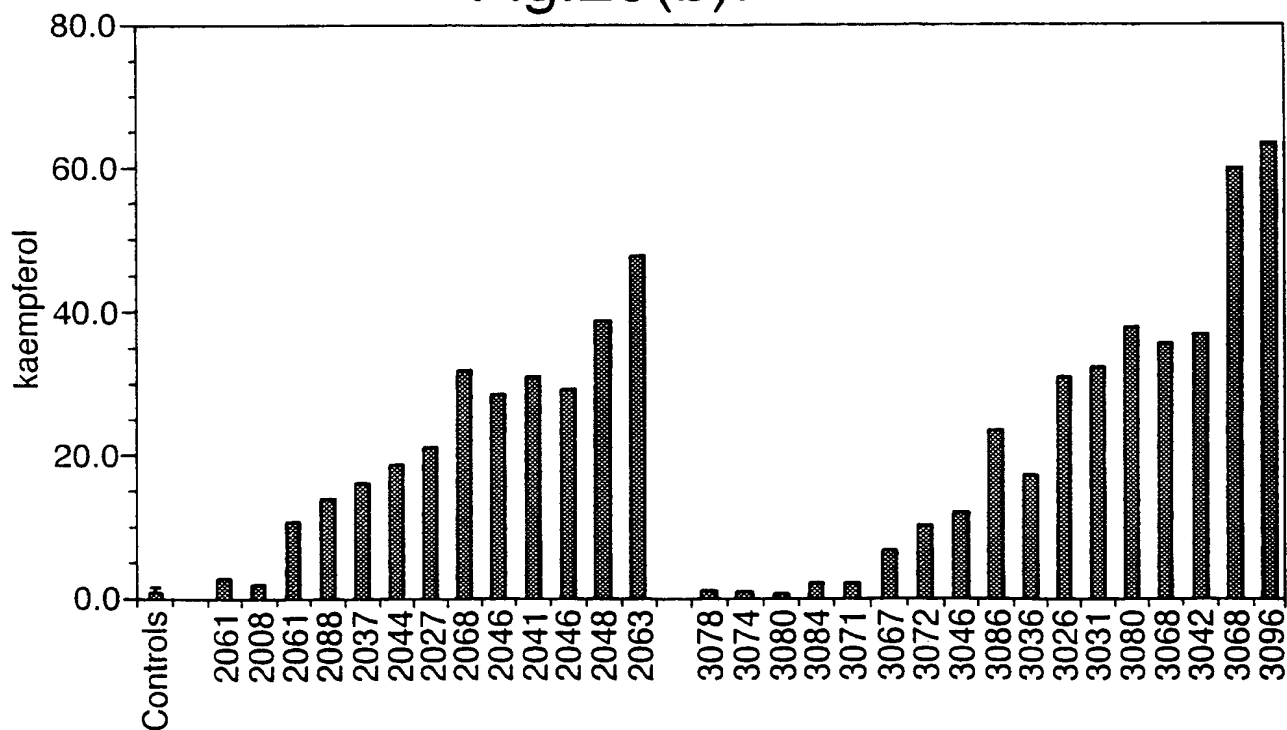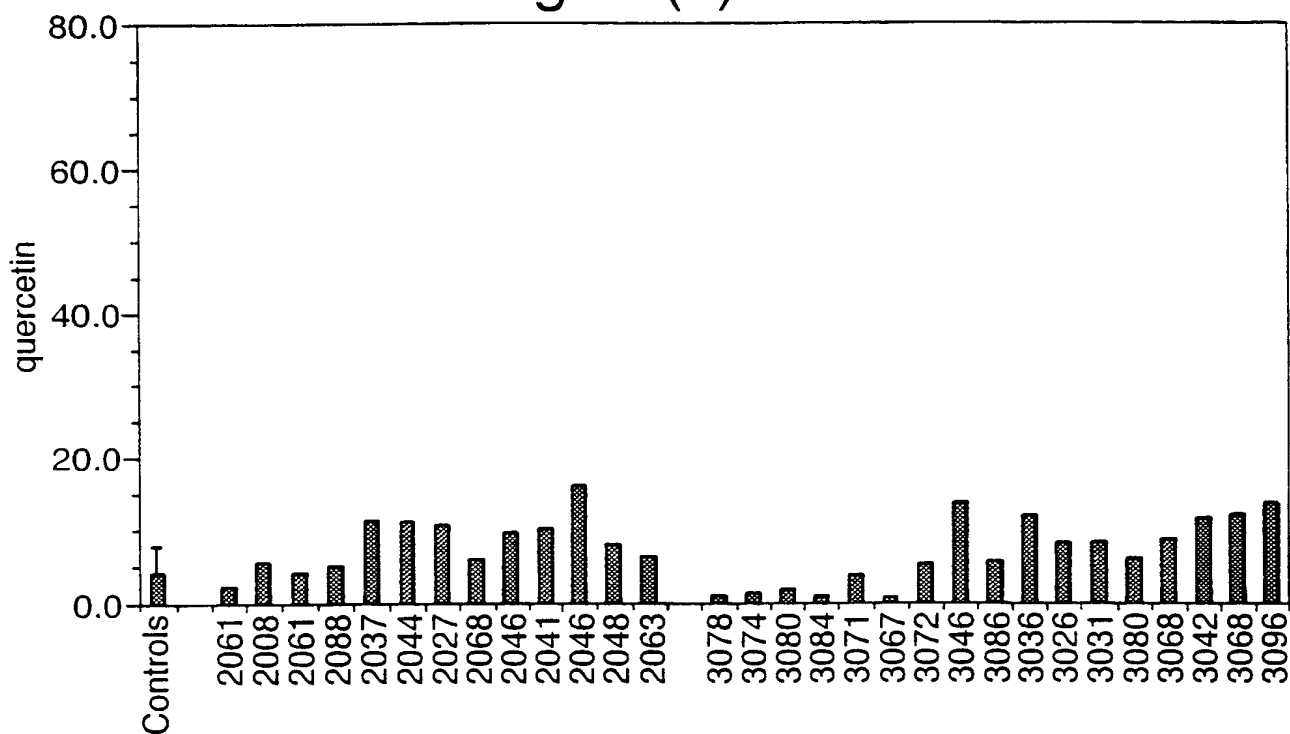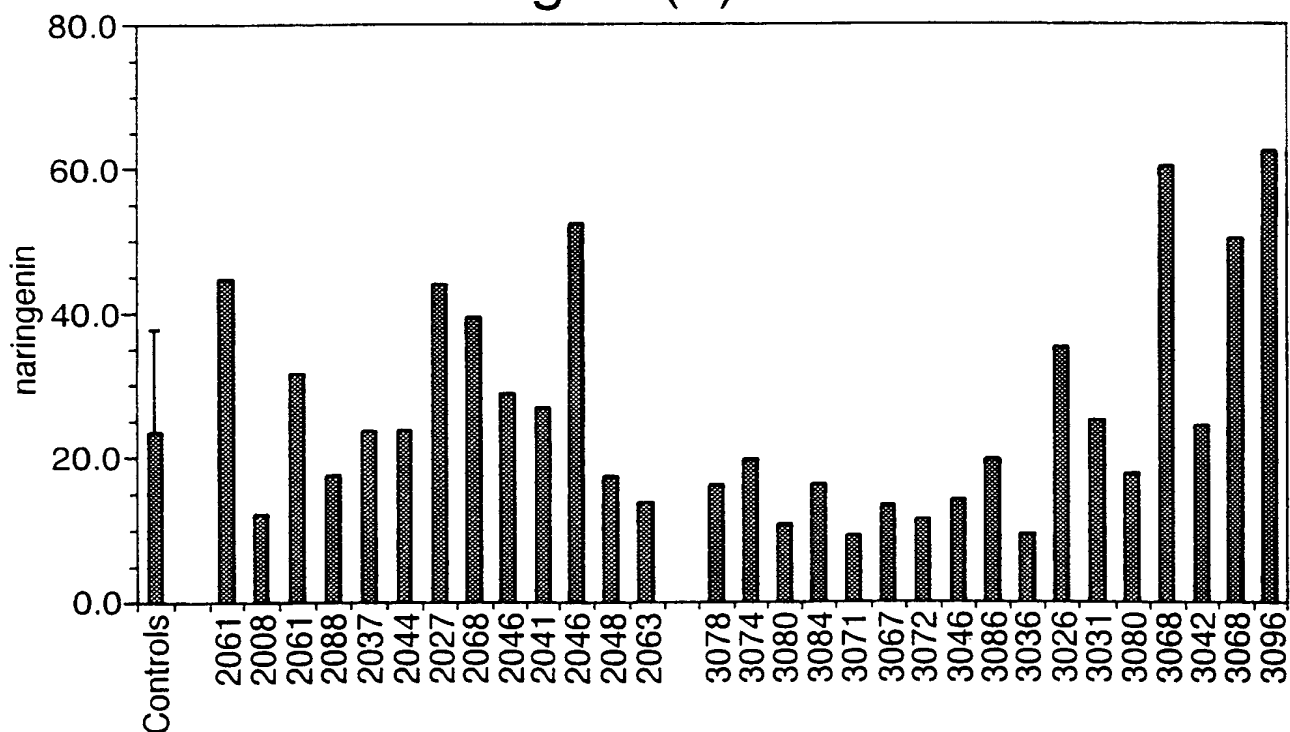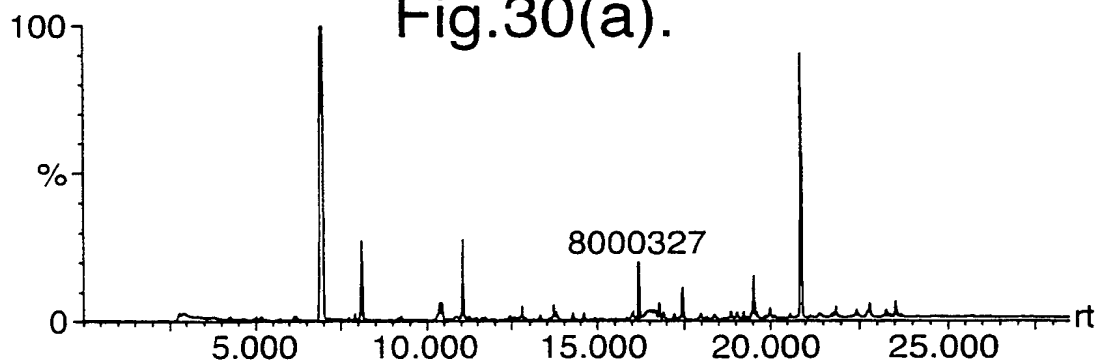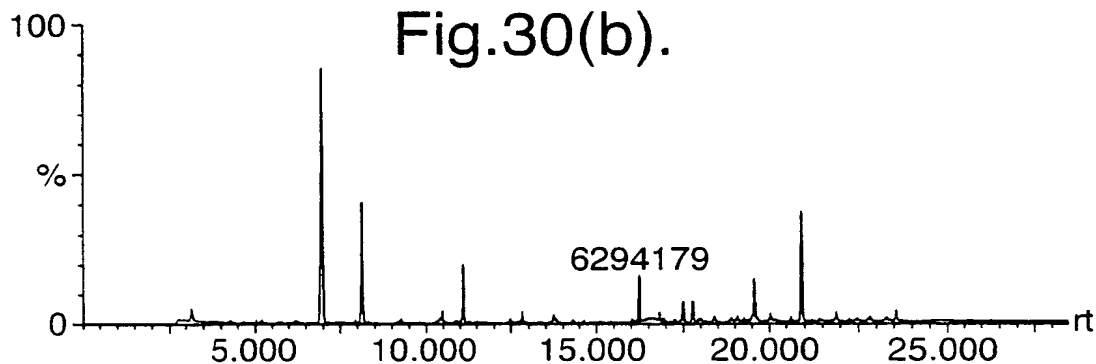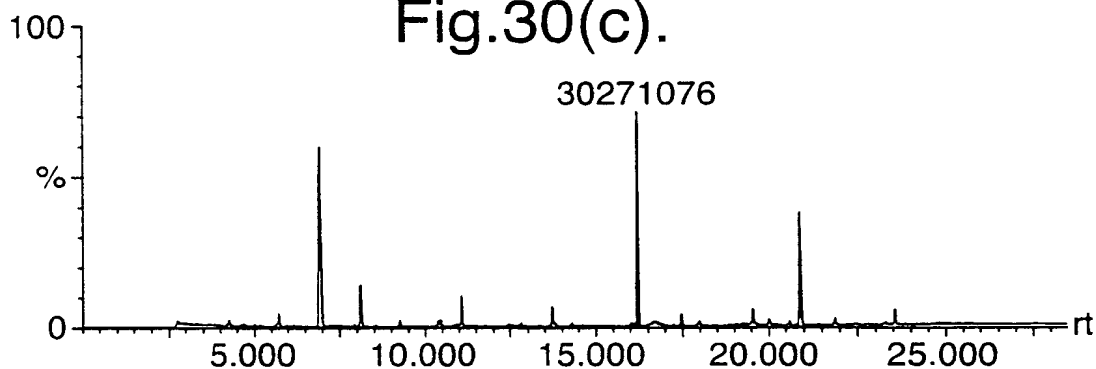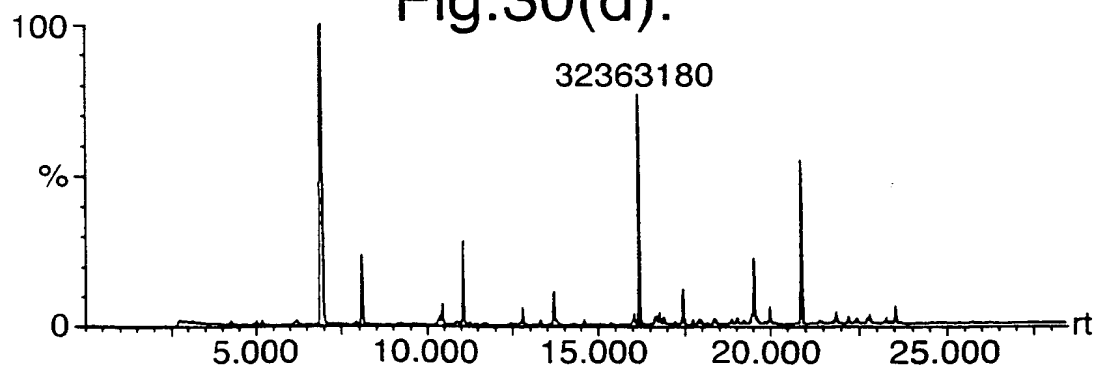
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12N15/82   C12N15/29   A23L1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   C12N   A23L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 34634 A (LEEMANS JAN ;WILLIAMS MARK (BE); PLANT GENETIC SYSTEMS NV (BE); KR) 21 December 1995 (1995-12-21) page 47, line 25 - page 51, line 4 ---	11-16,18
X	LLOYD, A.M., ET AL.: "Arabidopsis and Nicotiana anthocyanin production activated by maize regulators R and C1" SCIENCE, vol. 258, 11 December 1992 (1992-12-11), pages 1773-1775, XP002106592 cited in the application the whole document --- -/--	11,12,16

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
18 June 1999	06/07/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl. Fax: (+31-70) 340-3016	Maddox, A

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